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**SYNTHESIS AND ANTIBACTERIAL ASSESSMENT OF WATER-SOLUBLE
HYDROPHOBIC CHITOSAN DERIVATIVES BEARING QUATERNARY
AMMONIUM FUNCTIONALITY**

**A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural & Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy**

in

The Department of Chemistry

**by
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December 2004**

To my wife, *Prachee* for her love and support
and
my *Parents, Grandmother* and *Brother* for their love and best wishes

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ABSTRACT

Chitosan is a naturally occurring, bio-degradable, non-toxic, non-allergenic bio-polysaccharide derived from chitin, found in abundance in nature. Studies aimed at deriving newer applications of chitosan are hence of interest. Among these, one of the focuses of investigations is aimed at excellent antimicrobial activity demonstrated by chitosan. In this context, certain quaternary ammonium derivatives of chitosan have been shown in the past to be highly effective antibacterial materials. In the present study, substituted chitosan derivatives were synthesized and further quaternized using Quat-188 to produce water soluble derivatives at neutral pH. The antibacterial assessments of the quaternized derivatives of substituted chitosan were performed on liquid cultures of *E. coli* and *S. aureus* and results expressed in terms of Minimum Inhibitory Concentration (MIC). A comparison with the activity exhibited by the unsubstituted chitosan quaternized derivative indicated that hydrophobic substituents greatly enhance the activity.

Three synthetic routes, viz. Bosch reduction, γ -lactone addition and an anhydride addition were used to obtain hydrophobic as well as strongly and weakly acidic organic groups. In the case of Bosch reduction using aromatic aldehydes, a logarithmic relationship was observed between the %substitutions targeted and %substitutions obtained. *n*-Octyl aldehyde showed linear correlation between targeted and obtained %substitutions only in the presence of dimethyl formamide. γ -Octanoic lactone addition resulted in very low levels of substitution (<1.5% with up to 30% feed). However, the derivatives obtained using this method provided excellent antibacterial activity (MIC=16 μ g/mL, compared to 128 μ g/mL for the unsubstituted quaternized chitosan).

Heptanoic anhydride produced materials with 1.5-2.4% substitution which demonstrated high antibacterial activity (MIC=32 μ g/mL); however no substitution control could be realized using anhydride route. γ -Lactone addition and anhydride addition are simple methods of synthesizing chitosan derivatives with potential applications in industry.

Chitosan derived gels were prepared using an aromatic, rigid (terephthaldicarboxaldehyde) and an aliphatic, flexible (glutaraldehyde) cross-linkers and their quaternized derivatives exhibited antibacterial activity at low levels of cross-linking. Additionally, gels with rigid cross-linker exhibited enhanced activity compared to the flexible cross-linker. The onset of gelation for the aromatic gels was found to vary with the extent of the presence of cross-linker in gels (4.5mg/mL for 4.8% and 3.0mg/mL for 49%).

CHAPTER 1. INTRODUCTION

In 1811, Prof. Henri Braconnot, Director of the biological garden at Nancy, France isolated a fibrous substance from certain type of mushroom. He further observed that this substance did not dissolve in aqueous acidic solutions, e.g. sulfuric acid. A decade later, the same substance was found to be present in certain insects as well and in 1823 Ojean (sometimes Odier) named it 'Chitin' (from Greek *khitōn* meaning envelope). In 1859, Prof. C. Rouget subjected chitin to alkali treatment and observed that unlike chitin, the substance resulting after alkali treatment dissolved in acids; however, it was not until 1894 that this substance was named 'Chitosan' by Hoppe-Seyler. In the mean time, in 1878, Ledderhose proposed chitin to be made of glucosamine and acetic acid.

During 1930's and 1940's these biopolymers of glucosamine gained much interest within the oriental world, mainly in applications in the field of medicine and water purification. During 1970's the interest in these bio-macromolecules renewed at a brisk pace resulting in the first ever Chitin-Chitosan conference being held in the United States in 1977. Pioneering work of Muzzarelli during 1980's has greatly advanced our understanding of these materials. Today we know that chitin and chitosan (collectively called as chitineous substances) are found in abundance in nature and are renewable sources;¹ and this very fact has attracted much interest in developing new applications from these simple substances. In addition, chitineous polymers are biocompatible, biodegradable, non-toxic materials and this has expanded the possibility for the use of their derivatives in applications in medicine.² One of the striking differences between chitineous bio-polymers and certain semi-synthetic cellulose derivatives is the nitrogen content (6.89% and approx. 1.25% respectively) which can have a direct effect on

specific applications such as a chelating agent in waste water treatment.³ The greater nitrogen content in chitinous biopolymers, especially chitosan, also makes available numerous nucleophilic sites on the polymer backbone, compared to say, cellulose. These sites provide means of easier and selective synthetic transformations of these polymers to obtain various derivatives as may be necessary towards developing targeted applications. The annual production of chitin in nature is estimated at 10^9 - 10^{10} tons and currently it stands under utilized and under-commercialized compared to the cellulose industry.⁴

1.1 Background

Chitin is the second most abundant polysaccharide in nature, second only to cellulose; and is primarily present in the exoskeletons of crustaceans (such as crab, shrimp, lobster etc.). In addition to crustaceans, it is also found in various insects, worms, fungi and mushrooms, in varying proportions from species to species and from region to region (Table 1).⁵

Table 1: Approximate chitin content in various living species⁵

Species	Weight % chitin by dry body weight
Fungi	5-20%
Worms	20-38%
Squids/Octopus	3-20%
Scorpions	30%
Spiders	38%
Cockroaches	35%
Water Beetle	37%
Silk Worm	44%
Hermit Crab	69%
Edible Crab	70%

Chemical structure of chitin consists of linear repeating units of 2-acetamido-2-deoxy-D-glucopyranose attached through β -(1 \rightarrow 4) linkages (Figure 1a). The purpose it serves in nature is that of a structural polysaccharide, providing adhesion between fiber beds of the stacked laminae. For example, in the case of mollusk shells⁶, the shell matrix is composed of two structural units (Figure 2, schematic depiction); the first is a high molecular weight chitinoproteic complex arranged in the form of sheets and layers. The second unit, called mineralization matrix is a polypeptide with a strong affinity for calcium. During the shell growth, chitinoproteic complex remains trapped between the mineralization matrices providing means of adhesion between the two adjacent matrices. This tightly packed assembly provides excellent structural rigidity and mechanical strength to the shells.

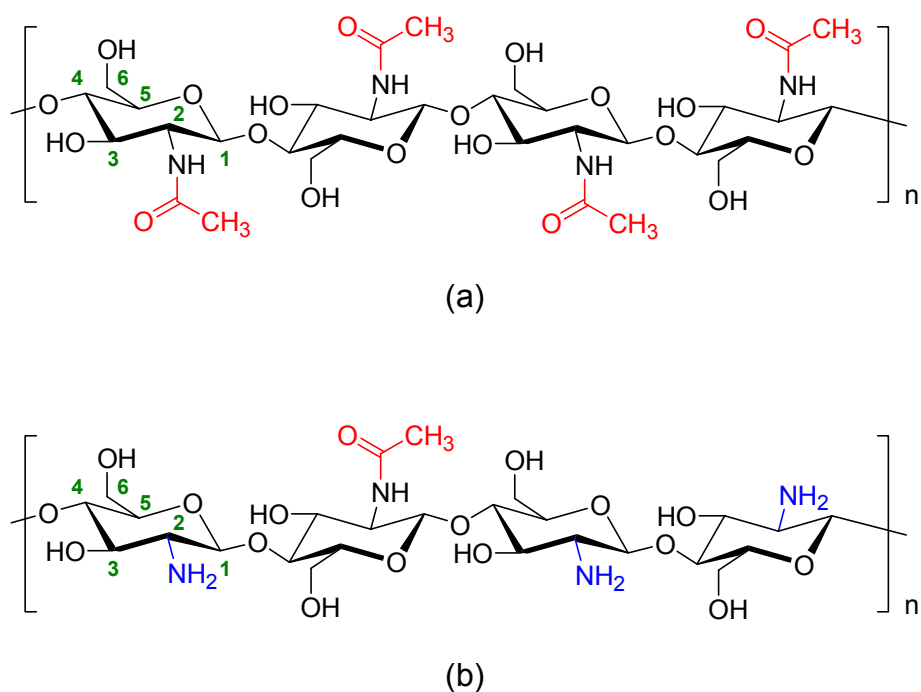


Figure 1: Chemical structures of chitin (a) and chitosan (b)



Figure 2: Mollusk shell schematic

Chitosan (Figure 1b) is deacetylated chitin; a structural modification of chitin often performed by alkaline hydrolysis. The actual process involved during the hydrolysis governs the extent of deacetylation in chitin, and accordingly, commercially available samples of chitosan contain anywhere from 70% to 100% deacetylation.⁷ In addition to the degree of deacetylation for a given chitosan sample, it is also characterized by the molecular weight of the macromolecule, and is often available within the range of 150,000 – 600,000. Chitin and chitosan are of high commercial interest due to their high nitrogen content (6.89%), which was first exploited in chelating agents.³ Additionally, both chitin and chitosan are biodegradable, biocompatible, non-toxic, nonallergenic, renewable biomaterials and find applications in fields such as medicine, perfume and cosmetics, food industry and agriculture.^{8,12} Chitin is highly hydrophobic and insoluble in water, acids, bases and common organic solvents. Some of the organic solvents in which it is soluble are hexafluoroisopropanol, hexafluoroacetone, chloroalcohols in presence of aqueous mineral acids and dimethyl acetamide in presence of 5% LiCl.⁹ Chitosan on the other hand, due to the presence of primary amino group in most of the sugar units that make up the polymer backbone, dissolves in dilute organic acids, but is insoluble in water at or above pH 6-7 and in commonly used organic solvents. The solubility of chitineous substances is usually associated with the crystallinity of the

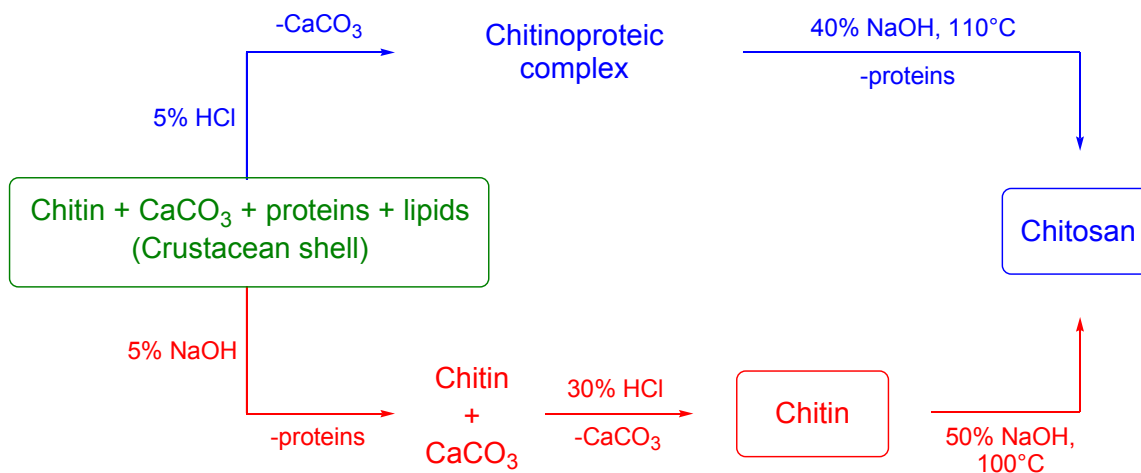
sample.¹⁰ Higher crystallinity suggests greater order and enhanced molecular interactions within and between the polymer chains. A chitinous substance can only dissolve under conditions when these interactions are disrupted. Intra and inter-molecular hydrogen bonding within the polymer chains is one major source of these interactions and it plays a major role in the low solubility of these substances. However, chemical modifications of chitosan have resulted into such derivatives that are water soluble over a wider range of pH, including strongly basic mediums. These modifications primarily introduce ionic groups (or substituents) into the polymer backbone, which are solvated by polar solvents like water by polar-polar interactions and help promote solubility of the macromolecule.

1.2 Isolation of Chitin and Synthesis of Chitosan

Within its natural resources of commercial interest, chitin exists not as a stand-alone biopolymer, but rather in conglomeration with other biomaterials, mainly proteins, lipids, and inorganic salts. The isolation process of chitin starts at the sea-food industry (Scheme 1).¹¹ One of the by-products of this industry, *viz.* shells from crab, shrimp, etc. are first crushed into a pulverous powder to help make a greater surface area available for the heterogeneous processes to follow. An initial treatment of the shell with 5% sodium hydroxide dissolves various proteins, leaving behind chitin, lipids and calcium salts (mainly as CaCO_3). Treatment with 30% hydrochloric acid hydrolyzes lipids; dissolves calcium salts (demineralization) and other minor inorganic constituents. Chitin thus obtained can be hydrolyzed using 50% sodium hydroxide at high temperature to provide chitosan. Alternatively, if isolation of chitin is not desired, the acid-base sequence may be reversed to directly produce chitosan. In this method, crushed shells are first treated with 5% hydrochloric acid to remove calcium salts. This is then followed by protein and

lipids removal by the treatment with 40% sodium hydroxide at higher temperature.

During the base treatment a concomitant hydrolysis of acetamido groups in chitin takes place, resulting in the formation of chitosan.



Scheme 1: Isolation of chitin and synthesis of chitosan– process schematic

In the United States, the production of chitin and chitosan is mainly carried out at the sea-food canning industry in the states of Oregon, Washington and Virginia. Other leading countries producing these bio-polysaccharides from similar sources are China, Japan, Norway, Mexico and Chile. Current processes utilize 6.3 kg of HCl and 1.8 kg of NaOH in production of 1 kg of 70% deacetylated chitin from shrimp shells.¹² Other factors controlling the cost are availability of water (for use in the process as well as a heat exchange medium), nitrogen gas (used to create inert atmosphere), transportation, energy costs and labor. In addition, recent advances in fermentation technology suggest that the cultivation of fungi (*Aspergillus niger*) can provide an alternative source of chitosan.¹³

Physical properties of chitineous substances are governed, at large, by two factors; the degree of deacetylation and the molecular weight. Degree of deacetylation has a direct

impact on the secondary structure of the polymeric chain and can also influence the solubility of the polymer in organic or aqueous solvents. It can thus affect the chemical reactivity of the sample in homogeneous processes. As an evolved nomenclature, chitineous substances that do not dissolve in dilute organic acids, e.g. 1-2% acetic acid, are collectively called chitin. Such polymers tend to have a low degree of deacetylation. On the other hand, chitineous substances that can dissolve in aqueous dilute acids are referred as chitosan. It is often past 60% deacetylation that aqueous solubility in mildly acidic solutions is realized. However, at levels of deacetylation between 50-60%, the distribution of remnant acetyl groups along the polymer chain greatly influences the solubility of sample. A random distribution of acetyl groups along the polymer backbone often results under homogeneous processing conditions, and renders such polymers soluble in aqueous solutions of weak acids.¹⁴ On the contrary, under heterogeneous processing conditions, polymers are formed with distinct blocks of acetylated sugar residues and show poor to no solubility in similar solvents.

Molecular weight of the chitosan obtained at the end of the production process depends on process parameters such as time, temperature and concentration of HCl and NaOH used. The process parameters used in the production of chitosan are quite drastic; and the process usually accompanies with scission of the parent chitin backbone. The degradation of the parent chitin chain could sometimes be extensive. In one such preparation, a chitin sample with weight average MW of 1.03×10^6 kDa resulted into chitosan with weight average MW 1×10^5 kDa.¹⁵ The MW determination of chitosan samples can be performed by various techniques such as light scattering spectroscopy¹⁶, viscometry¹⁷ and gel permeation chromatography¹⁸. Viscometry is relatively simple

technique and uses the Mark-Houwink relationship (Equation 1) in the determination of the MW. The constants K and α are well documented in literature for dilute chitosan solutions in aqueous 0.1 M acetic acid/0.2 M sodium chloride solvent. However, the charged nature of chitosan, its tendency to form self-aggregates and differences in the degrees of deacetylation for different chitosan samples require careful implementation of the constants in Equation 1.

Equation 1
$$[\eta] = K \overline{M}_v^\alpha$$

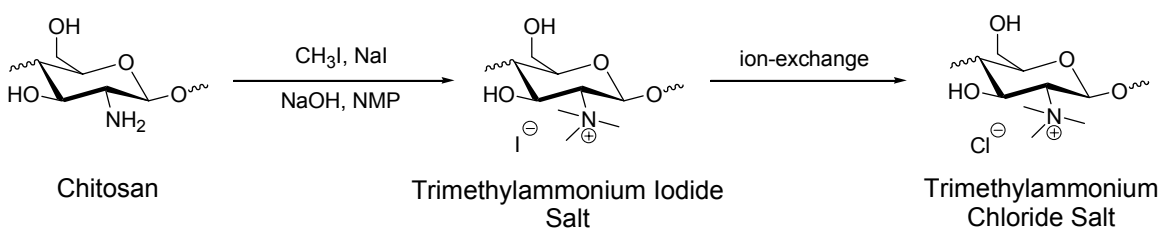
1.3 Water-soluble Derivatives of Chitosan at Neutral pH

Most of the real life applications for any chemical substance, whether natural or synthetic in origin, require the chemical to be processible. In this regard chitosan, a white flaky solid, is difficult to manipulate with because of the solubility problems in neutral water, bases, and commonly used organic solvents. The pK_a value of the primary amino groups in chitosan is determined to be around 6.5.¹⁹ As a result, even though chitosan and its derivatives are soluble in pH values of lower than 6.0, many of its applications in neutral or basic medium, including those of physiological relevance, may not be realized, for the pH under such situations will trigger an immediate precipitation. On the other hand, acidic solutions, in which chitosan is fairly soluble, may not be desirable in many of its applications, especially those in medicine, cosmetics, and food. There have been two major approaches documented in literature towards improving the solubility of chitosan at neutral pH. First is to chemically derivatize chitosan²⁰ (for example with substituents containing quaternary amino groups, carboxymethylation, and sulfatation) such that the substituent added is strongly hydrophilic. The second approach uses

chitosan with an average 50% deacetylation prepared by homogenous processing of chitin.¹⁴ Under the homogeneous processing conditions, chitosan obtained remains solubilized upon neutralization and thus needs no further derivatization if such is undesired. However, several of the applications of chitosan cited in the literature utilize some form of chitosan derivative and hence to provide for the solubility, it may be necessary to incorporate ionic groups into the polymer backbone.

1.3.1 Quaternized Derivatives of Chitineous Substances

The simplest derivative in this class is the trimethylammonium salt of chitosan. A repeated treatment of chitosan in *N*-methyl-2-pyrrolidone (NMP) containing sodium iodide and methyl iodide in presence of sodium hydroxide results into the trimethylammonium salt of chitosan with a high degree of substitution (Scheme 2).²¹ An anionic exchange of iodide with chloride ion may be necessary due to stability issues. The resulting product is water soluble at neutral pH.

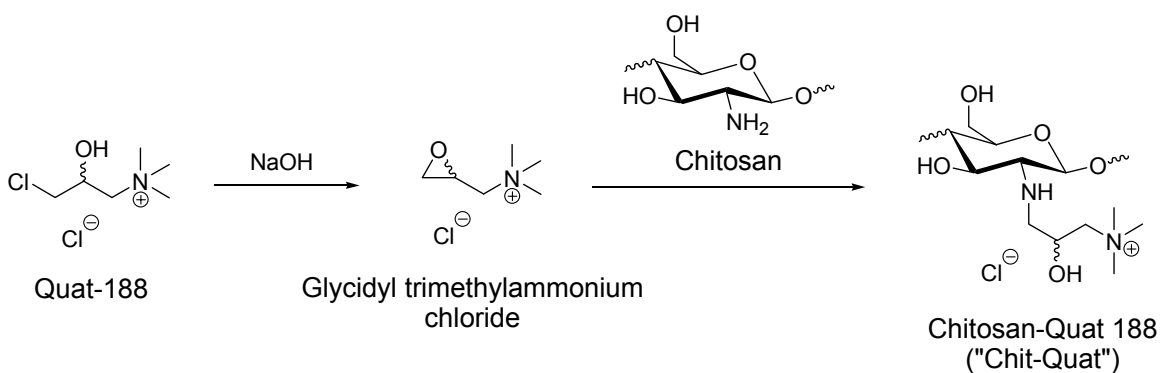


Scheme 2: Synthesis of trimethylammonium salt of chitosan

Lang *et al.* synthesized the quaternary ammonium salt using glycidyl trimethylammonium chloride as the quaternizing agent.²² In an alternate approach, Daly and Guerrini have developed a method using commercially available and stable Quat-188 salt [(3-chloro-2-hydroxy)propyltrimethylammonium chloride] in preparation of similar derivatives (Scheme 3).²³ The latter approach produces the glycidyl reagent *in situ* and

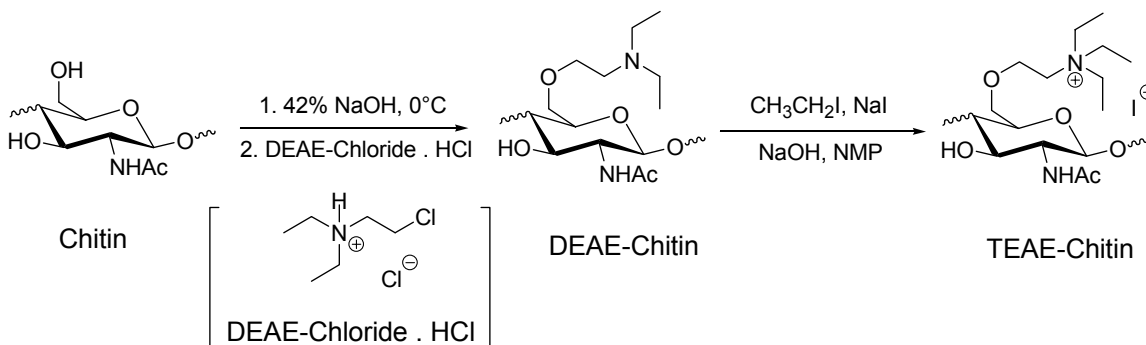
thereby reduces safety concerns associated with using epoxides in a large scale set up.

The derivative Chit-Quat shows excellent solubility in neutral water. The process of introducing quaternary ammonium substituents on the chitosan backbone will be referred hereafter as the process of ‘quaternization’. In this context, the ‘quaternizing agent’ referred to will always be Quat-188 (Scheme 3), unless otherwise specified.



Scheme 3: Synthesis of Chit-Quat from chitosan and Quat-188

Triethylaminoethyl derivative of chitin (TEAE-Chitin) shows good water solubility at neutral pH. This derivative was prepared by first activating the C-6 primary hydroxy group in chitin at low temperature, in order to minimize undesired deacetylation, if any (Scheme 4).²⁴

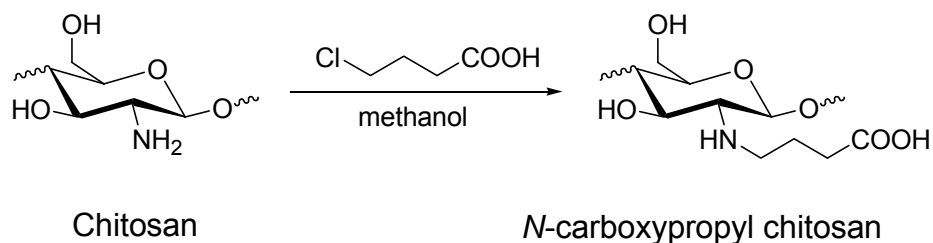


Scheme 4: Synthesis of triethylaminoethyl-chitin (TEAE-Chitin)

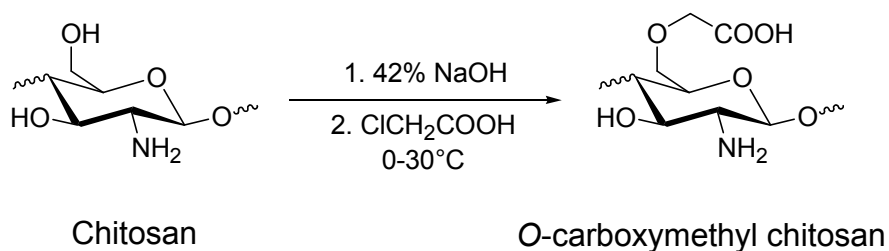
The activated chitin was then allowed to react with diethylaminoethyl chloride, as its hydrochloride salt followed by quaternization using ethyl iodide. Interestingly, the intermediate DEAE-Chitin derivative was also found soluble at neutral pH.

1.3.2 Carboxyalkylated Derivatives of Chitosan

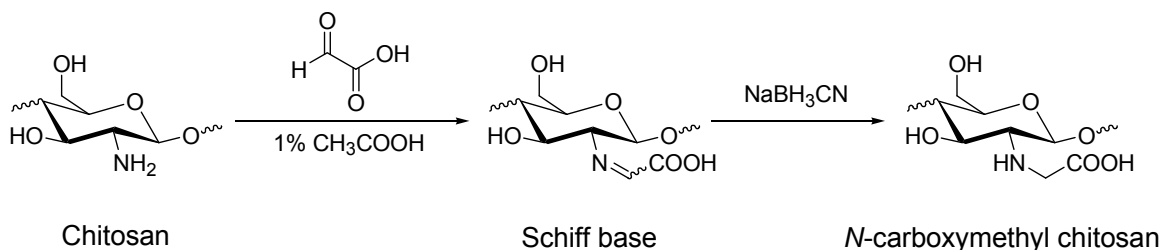
The process of carboxyalkylation introduces acidic groups on the polymer backbone. However, due to the presence of native amino groups in chitosan, these derivatives exhibit amphotericity. As a result, these derivatives exhibit an isoelectric point observed in other amphoteric molecules such as polypeptides, at which the polymer overall does not carry any net charge. At the isoelectric point pH the polymer remains undissolved. Water solubility however is attained at pH values above or below the isoelectric point. Both, *N*-carboxyalkyl and *O*-carboxyalkyl chitosan derivatives have been prepared using different reaction conditions to attain the *N* vs. *O* selectivity (Scheme 5²⁵ and Scheme 6³⁸).



Scheme 5: N-carboxypropylation of chitosan



Scheme 6: O-carboxymethylation of chitosan

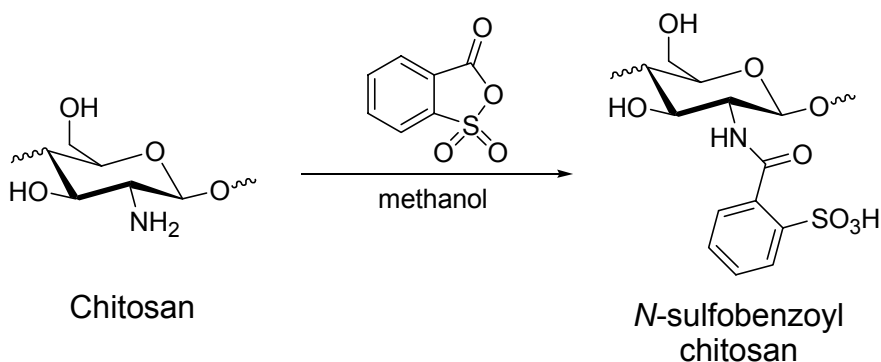


Scheme 7: *N*-carboxymethylation of chitosan using reductive amination approach

A third synthetic route that is selective in the formation of *N*-carboxyalkylation uses carboxyaldehydes in a reductive amination sequence (Scheme 7).²⁶ The reaction is carried out under homogeneous conditions, provided that the aldehyde used is water soluble, and this allows for greater degree of substitution which, in principle, should be well distributed along the polymer backbone. However, sequential substitutions giving rise to the formation of bis-carboxymethyl derivatives have been observed using glyoxylic acid.²⁷

1.3.3 *N*-sulfonated Derivatives of Chitosan

Similar to *N*-carboxyalkylated derivatives of chitosan, *N*-sulfonated derivatives are amphoteric in nature. They can be prepared under heterogeneous reaction conditions using 2-sulfobenzoyl acid anhydride (Scheme 8).²⁸



Scheme 8: Synthesis of *N*-sulfobenzoyl derivative of chitosan

1.3.4 Miscellaneous Derivatives

In addition to the ionic derivatives mentioned earlier, a few non-ionic, polar derivatives of chitosan have been prepared which show good water solubility at neutral pH conditions. The polar substituent used is either of carbohydrate origin²⁹ (with several hydrophilic hydroxy groups; Figure 3a, b), or a graft of polar polymer on the chitosan backbone³⁰ (for example a graft of polyacrylic acid and various polar polyacrylates; Scheme 9). The structure of the resulting graft was not provided by the authors. However, by comparison with one report³¹, the most probable graft structure is given in Scheme 9.

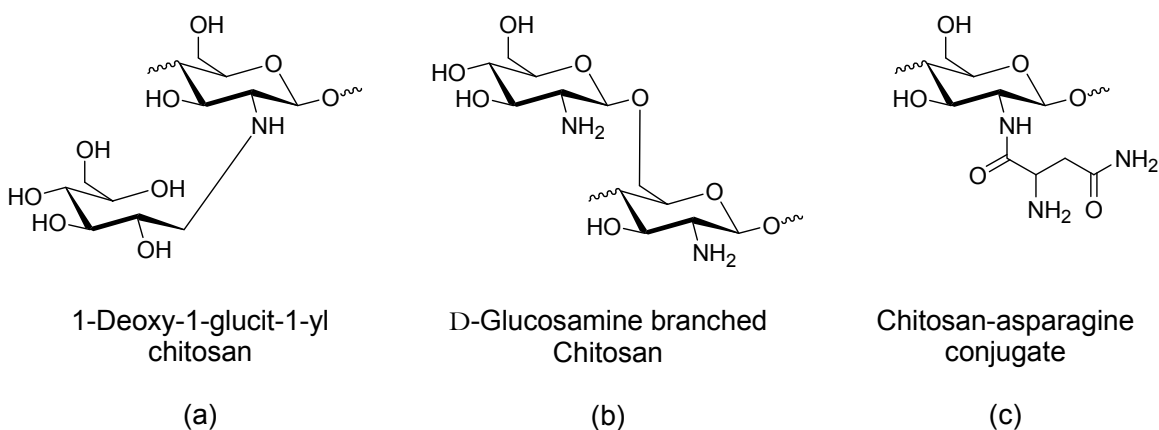
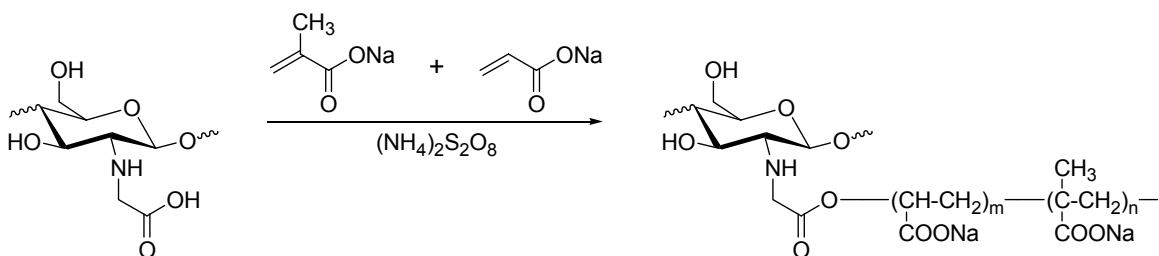


Figure 3: Chitosan derivatives with carbohydrate [(a), (b)] and amino acid [(c)] substituents



Additionally, amino acid substituent with ionizable sidechains (such as amino and carboxylic acid groups; Figure 3c) has also resulted in good solubility of such derivative in neutral water.³²

1.4 Applications of Chitosan

Traditionally, chitineous substances were used mainly in rudimentary medicine and waste water treatment. In past 2-3 decades these substances, predominantly derivatives of chitosan, have found applications in diverse fields, varying from tissue engineering to photography. In this context, chitosan and its derivatives being soluble under aqueous conditions have been the major focus of interest compared to chitin. However, chitin and its derivatives have found applications in medicine, for example in wound dressings and absorbable sutures made of chitin fibers. Of particular interest is the resistance of chitineous substances towards bile, pancreatic juices and urine.³³ This allows for their application in surgical sutures within these areas of human body where sutures made of other materials may be prone to attack by the bodily fluids prior to the healing process. In addition, chitin has been used as the raw material in man-made fibers.³⁴ Chitineous substances can undergo degradation with *lysozyme*; an enzyme found in common in nature and in human eyes³⁵, and by *chitinases*.³⁶ This has allowed for the use of chitosan derivatives in contact lens cleaning solution for effective removal of enzyme deposits.³⁷ Specific applications of chitosan are summarized below by the application type.

1.4.1 Antibacterial Properties

Chitosan and several of its derivatives show good to excellent antimicrobial (antibacterial and antifungal) property (Table 2; due to chitosan).³⁸ The antibacterial

action is usually rapid and eliminates bacteria as quickly as within few hours.³⁹

Furthermore, chitosan derivatives are biodegradable and show low toxicity towards mammalian cells.⁴⁰ The activity is wide spectrum and includes bacteria of both cell wall types; Gram positive and Gram negative. It is however important to note that the monomer of chitosan, 2-amino-2-deoxy-D-glucopyranose as its hydrochloride salt, does not exhibit any antibacterial activity against several bacteria, including *E. coli* and *S. aureus*.⁴¹ The antibacterial activity thus seems to be associated with the chain length of the polymer and suggests a cooperative effect of individual sugar units. In comparison, chitin does not show any antimicrobial activity.⁴² The antibacterial property of chitosan is particularly useful in the field of medicine where it can be used to make surgical accessories such as, gloves, bandages etc. It has also been used in the removal of water-borne pathogens in waste water and as a food preservative by applying a coat on the exterior of vegetable and fruit produce.⁶

Table 2: Antibacterial and antifungal activity of chitosan^{44a,38}

<i>Bacteria</i>	MIC (ppm)	<i>Fungi</i>	MIC (ppm)
<i>Agrobacterium tumefaciens</i>	100	<i>Drechsteria sorokian</i>	100
<i>Erwinia carotovora ssp.</i>	200	<i>Rhizoctonia solani</i>	1000
<i>Pseudomonas fluorescens</i>	500	<i>Trichophyton equinum</i>	2500
<i>Staphylococcus aureus</i>	20	<i>Botrytis cinerea</i>	10
<i>Micrococcus luteus</i>	20	<i>Fusarium oxysporum</i>	10

Chitosan blended with polyester and cellulose fibers render antibacterial property to such fibers.⁴³ The resulting fibers show excellent laundering durability and retention of antibacterial activity following several laundering cycles.⁴⁴

1.4.2 Application in Cosmetics and Artificial Skin and Dressings

Chitosan is the only naturally occurring cationic gum which turns viscous upon dissolution in mildly acidic medium.⁴⁵ This property has been used in various cosmetic applications, particularly in hair products and setting agents, hair conditioners and shampoo.⁴⁶ Fibers grafted with chitosan make good dressing material. In addition to the antibacterial activity of chitosan, which helps keep wound sterile, it is also shown to aid in the healing process in common wounds, thus showing biological activity.⁴⁷ The antimicrobial property has spurred an interest in deriving such materials incorporating chitosan as an integral part and targeted for different applications. For example, materials resulting from co-spinning of rayon and chitosan have shown good antimicrobial activity and are demonstrated to serve as excellent bandage materials for the treatment of common wounds. In addition, chitosan is shown to play a direct role in actual wound healing process in animals by acting as a stimulant for polymorphonuclear leukocytes (PMN).⁴⁷ The latter shows an enhanced phagocytic activity upon interaction with chitosan, which in turn causes an increase in the production of bactericidal oxygen species at cellular level. Application of chitosan grafted materials in biomedical gloves, fabrics is very attractive. Several reports in literature have demonstrated preparation and usage of artificial skin and dressing materials incorporating chitosan.⁴⁸

1.4.3 Waste-water Treatment

Chitosan, owing to the presence of amino groups within its backbone effectively binds various metal ions. Chitosan and several of its water insoluble derivatives have shown excellent binding capacities against toxic and hazardous heavy metal ions such as Sn^{2+} and Sn^{4+} (including organic tins), Hg^{2+} , Pb^{2+} , U^{6+} ; and transition metal ions e.g. Cd^{2+} ,

Cu^{2+} , Cr^{3+} , Zn^{2+} , Ni^{2+} , V^{4+} etc.⁴⁹ The metal adsorption capacity seems to depend on the particle size and amount of chitosan present in the adsorbing material, and on the concentration of metal ions in solution from which they are adsorbed.⁵⁰ Antimicrobial activity of chitosan has been shown useful in removing waterborne pathogens.⁵¹

1.4.4 Miscellaneous Applications

The film forming ability, resistance to abrasion and optical characteristics of chitosan has resulted in its use in photographic film.⁵² Chitosan has also been used as food preservative and coatings of chitosan have shown to extend the life of produce goods.⁵³ Further, since chitosan is completely biodegradable and non-toxic, its use does not produce adverse effects on humans or the environment. Chitosan has been used as a chromatographic support for separation of nucleic acids.⁵⁴ In this context, the amino group and hydroxy groups in chitosan act as affinity sites for polar materials. Chitosan has also been shown useful in diet of farm animals and birds. Some of the recent applications of chitosan have looked at materials made of chitosan with the targeted use as non-linear optical material (NLO).³¹

1.5 Present Project

Chitosan and its derivatives have great potential as effective and inexpensive antimicrobial agents. Of particular interest to us are the derivatives of chitosan that contain quaternary ammonium groups in the polymer backbone. There have been numerous reports in literature along with the past experience within the research group of Prof. W. H. Daly, suggesting a vital role of quaternary ammonium group in rendering antimicrobial activity to chitosan derivatives.⁵⁵ Some of the previous work conducted

within the Daly group, using such derivatives of substituted chitosan, has shown excellent antibacterial activity (as low as 32 ppm against *E. coli* and *S. aureus*) due to these derivatives.²⁷ When the activity of Chit-Quat (Scheme 3) was assessed against *Mycobacterium tuberculosis*, it exhibited enhanced activity in presence of a hydrophobic adjuvant (phytol) as against when it was tested alone. This observation in addition to the fact that mycobacterial cell surface is composed of several hydrophobic domains suggest possible role of hydrophobic-hydrophobic interactions between the antibacterial compound and the bacterial cell wall during the course of activity. Bacteria other than mycobacterium, e.g. Gram positive and Gram negative bacteria are also known to contain such domains of hydrophobic residues; albeit the bacterial cell wall even though structurally complex, has a bilayer structure with non-polar, hydrophobic groups making up the core of the cell wall or membrane.⁵⁶

One of the logical deductions that can be drawn from these observations is that, an antimicrobial compound containing fewer hydrophobic groups or lacking them altogether, could turn more potent by introducing such groups within its structural framework. The goal was to synthesize various chitosan derivatives containing groups with varying hydrophobicity and at varying levels of their presence within the parent macromolecule. The substituted chitosan materials once obtained were quaternized using Quat-188 as the quaternizing agent. The resulting quaternized derivatives of substituted chitosan were assessed for their antibacterial activity against *E. coli* and *S. aureus*, the representatives of the two classes of bacteria (Gram negative and Gram positive respectively). The results obtained during this research are described here in detail.

CHAPTER 2. SYNTHESIS OF *N*-SUBSTITUTED HYDROPHOBIC CHITOSAN DERIVATIVES

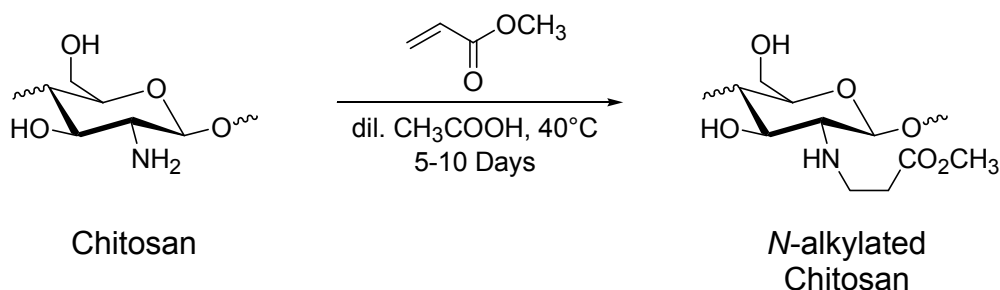
The structure of chitosan is useful to the synthetic organic chemist interested in site selective modifications. Such modifications have resulted into several derivatives of chitosan with distinct properties and applications. The presence of multiple nucleophilic groups within the chitosan backbone requires following suitable synthetic protocol in order to obtain the desired selectivity. The synthetic transformation steps performed are often relatively simple, exploiting the differences in the nucleophilicities of primary amino group (at C-2) versus the two hydroxy groups (at C-3 and C-6). However, the degree of substitution varies greatly upon the reaction conditions. Broadly speaking, the derivatives reported in literature mainly fall under one of the three categories; *N*-alkylation, *N* and/or *O* acylation and grafting of chitosan on a growing or preexisting polymer chain. These synthetic methods are briefly discussed here.

2.1 Literature Review

The synthetic modification of chitosan is a widely studied area. Several publications and patents have focused on derivatizing chitosan with a wide array of substituents. The derivatives are prepared for specific applications and hence the methods followed are based on the groups desired at the end of such transformations. As desired, there may be an additional “solubilizing” step involved within the synthetic route, where a polar ionizable substituent is attached to the derivative of chitosan.

2.1.1 *N*-alkylation of Chitosan

N-Alkylation of chitosan is selectively carried out using either a halogen displacement reaction performed on a organic halide (similar to Scheme 5)²⁵; a 1, 4 addition reaction with α - β unsaturated esters (Scheme 10)⁵⁷; or by using a reductive amination (Bosch reduction) approach (similar to Scheme 7)⁵⁸. The first approach is usually carried out under heterogeneous reaction conditions and normally results in lower degree substitution. The latter two approaches may be carried out under homogeneous reaction conditions and hence provide for higher degrees of substitution when desired. Since the primary amino group in chitosan is more nucleophilic compared to the hydroxy groups under neutral conditions, substitution takes place preferentially on nitrogen under equilibrium conditions.

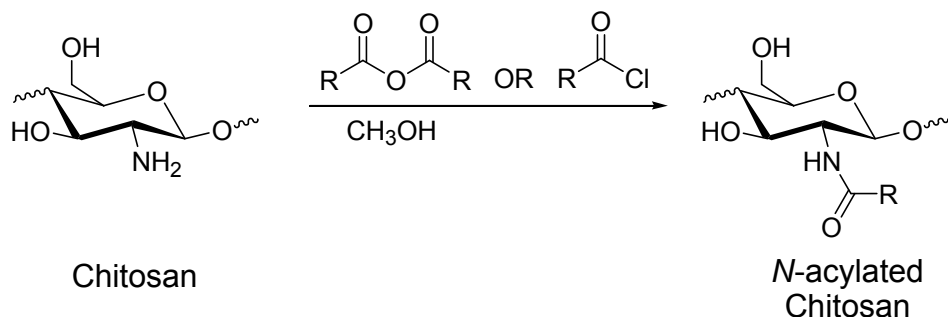


Scheme 10: *N*-alkylation via 1, 4-addition to α - β unsaturated esters

Various long chain alkyl substituents have resulted in chitosan derivatives with varying degree of hydrophobicity. Such materials are industrially important as they show unusual and interesting rheological properties thought to arise from the intermolecular association of neighboring hydrophobic substituents.⁵⁹

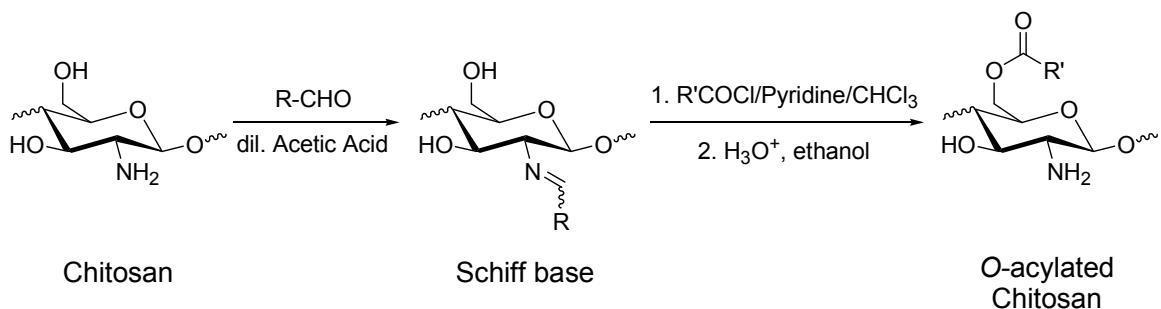
2.1.2 *N* and/or *O*-acylation

This is probably the most common way of derivatizing chitosan. The usual method involves reacting chitosan under heterogeneous reaction conditions with either an acid chloride or acid anhydride (Scheme 11)⁶⁰. There also has been a report for the preparation of a diimide derivative of chitosan using differently substituted phthalic anhydrides followed by thermal dehydration at elevated temperatures.⁶¹ Certain *N*-acyl chitosan derivatives cause selective aggregation of cancer cells and are therefore of interest.^{62,89} These derivatives also exhibit aggregation when dissolved in dilute aqueous acids.



Scheme 11: Synthesis of *N*-acyl derivatives of chitosan

Alkyl carbamates of chitosan were prepared by reacting methyl and ethyl chlorofomates with chitosan.⁶³ Acyl substitution was reported to take place on both *O* and *N* positions under a large excess of acid chloride.⁶⁴ Synthesis of *O* acylated chitosan was achieved by first protecting the primary group of chitosan as a Schiff base with suitable aldehydes (Scheme 12).⁶⁵ The Schiff base was allowed to swell in appropriate solvent and was reacted with acid chloride of choice under heterogeneous conditions. The amino group protection was removed in the last step by treatment with dilute aqueous acid to produce *O*-substituted acyl chitosan.

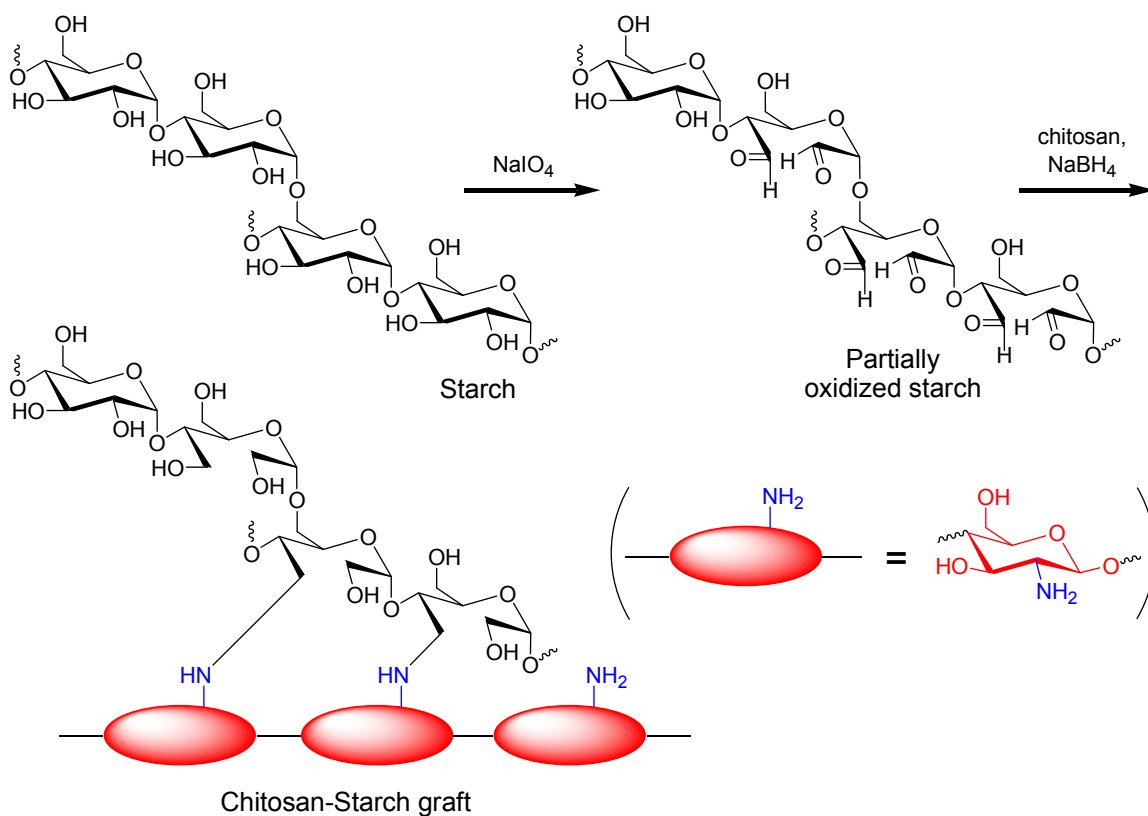


Scheme 12: O-acylation of chitosan using N-protection-deprotection route

When comparing the alkylation routes (section 2.1.1, *vide infra*) with the acylation route (section 2.1.2, *vide infra*), the latter seems more attractive from an industrial point of view. It uses readily available and inexpensive reagents, compared to, say NaBH_3CN , which could be undesirable due to the concerns related to safety, in addition to the cost factor of the process using it.

2.1.3 Chitosan Grafts

Chitosan grafts with other biopolymers (e.g. carbohydrate macromolecules) and synthetic polymers are carried out by solution phase chemistry (Scheme 13)⁶⁶ or under radical polymerization conditions (similar to Scheme 9)⁶⁷ respectively. The process of grafting produces materials with interesting properties and provides one possible means of advanced material development, where by physical and chemical properties of two different macromolecules are exploited. Chitosan and hyaluronic acid in different proportions form polyelectrolyte gel complexes which show excellent swelling properties.⁶⁸ These complexes are of particular interest since they rapidly attain the swelling equilibrium (≈ 30 min). It should be noted here that neither chitosan, nor hyaluronic acid, by itself demonstrates comparable swelling ability as against the polyelectrolyte complexes formed by the two.



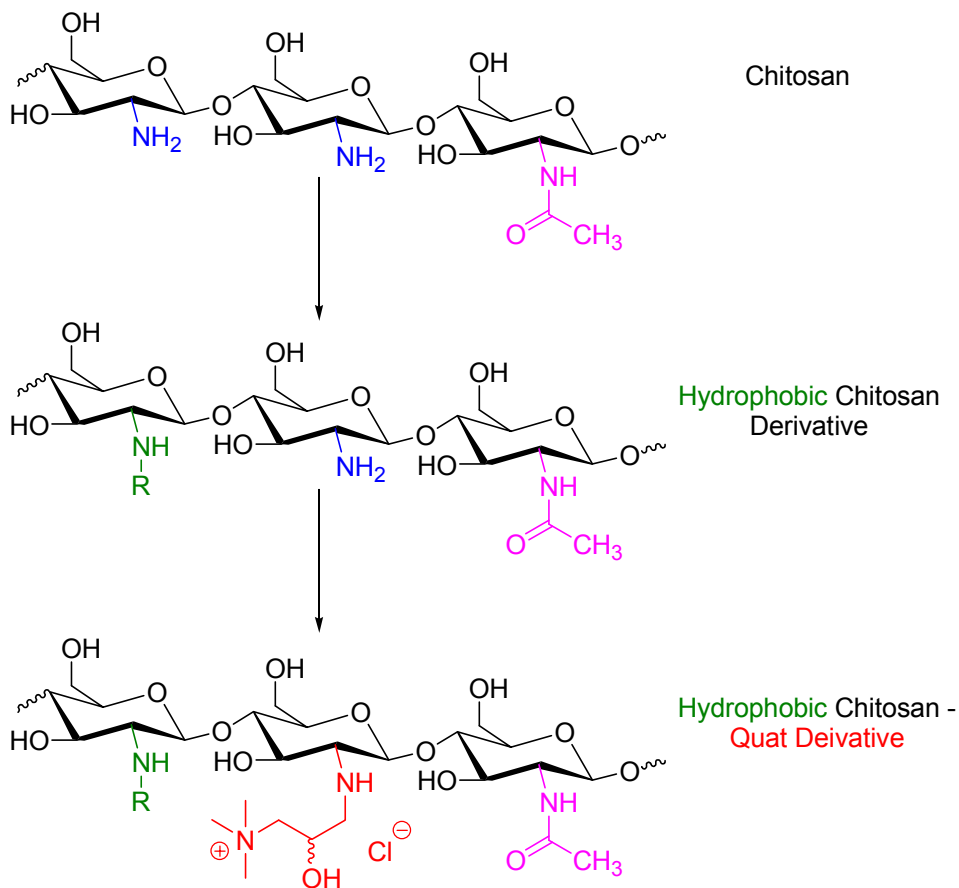
Scheme 13: Synthesis of chitosan-starch grafts

The water swelling ability of these complexes was found to be higher at higher levels of chitosan in the complex and was attributed as due to the hydrophilic nature of chitosan. Grafts of chitosan and polyacrylic acid have shown very high water-sorbing ability (≈ 600 w/w).⁶⁹ It was also observed that the swelling ability of the resulting graft depended upon the water content of the medium present during their synthesis.

2.2 Syntheses Performed in Present Project

An objective of the present project was in part synthesizing water-soluble hydrophobic chitosan derivatives bearing quaternary ammonium functionality. We approached this by following a two step protocol in which the first step involved synthesizing different derivatives of chitosan with varying degrees of hydrophobic character. Upon synthesis

and purification of such derivatives, they were further subjected to quaternization in a separate step to obtain targeted compounds (Scheme 14).



Scheme 14: Synthesis of hydrophobic chitosan-Quat derivatives

The nature of our project objective required selecting a versatile and well established method that can be used with various commercially available organic counterparts. We adopted three different synthetic approaches towards the synthesis of chitosan derivatives noting that they will result into different connectivity's for the substituent on the macromolecule chitosan. In addition, the reactions performed were carried out under mildly acidic conditions under which chitosan dissolves completely. The homogeneous conditions were selected to produce a statistically controlled random distribution of the

substituents along the macromolecule chain. The structural integrity of chitosan polysaccharide in dilute solutions of weak acids (e.g. acetic acid), but not in strong acids (e.g. HCl), is known to be good.^{58,70} All the synthetic methods used here are carried out using a solution of chitosan in 1% acetic acid, and even though we have not evaluated the extent of degradation of the parent macromolecule, we believe it to be miniscule. As a result, we assumed that DP of the macromolecule did not change substantially during the course of our syntheses.

The syntheses of various chitosan derivatives were performed, in most instances starting with one gram of chitosan. It was clear during the scaling up process of some of the derivatives that, the reaction times needed for the completion of a scaled up reaction in order to obtain similar results was longer than expected by simple linear extrapolation. For example, when twice the usual amount of chitosan were allowed to react with a representative γ -lactone, it required more than double the reaction time for the completion of reaction. Investigations aimed specifically at understanding the relationship between reaction scale and the time of completion of the reaction were not performed in this project.

2.2.1 Isolation and Purification of Products

Products when formed as a precipitate after the synthesis were filtered and washed with water to remove inorganics, followed by washings with copious amounts of acetone. It was observed that acetone not only washed away excess water and unreacted organic reagents, but also changed the texture and appearance of the product compared to when acetone was not used at all. The latter usually produced material that was intractable and rubbery, making it difficult to prepare samples for certain analyses. Acetone possibly

acts by removing water molecules trapped between two or more macromolecular chains, thus affecting inter-chain aggregation. This results in a substance that can be finely ground into a powder. It was noted that the material obtained in this fashion was comparable to the material obtained by more commonly used process of freeze-drying in the isolation of product. The only difference in the physical characteristics of the material obtained in either case being that freeze-drying usually produces very fluffy solid, where as acetone precipitation resulted in a dense, fine powder. However, compared to freeze-drying, precipitation with acetone was found economical, convenient and quick. ^1H NMR analyses of the products obtained by following either method were identical. After the acetone wash, products obtained were dried overnight at room temperature under a steady stream of nitrogen.

Quaternized derivatives of chitosan remained dissolved at neutral pH in the aqueous environment used in the course of their preparation. Purification of these derivatives involved an initial dialysis. The process of dialysis selectively removes small molecular weight impurities (inorganics, unreacted Quat-188, other low MW side products of the reaction etc.) by the process of diffusion, while the macromolecular products are retained within the boundaries of dialysis membrane due to their greater size.

2.3 Spectroscopic Analyses

^1H NMR spectroscopy was used in quantitative determination of degree of substitution. This method has been widely followed in literature for use in polymers and has an advantage in being quick, easy and reliable method.⁷⁰ The method has been studied for chitosan extensively and is shown to cause no degradation of the polymer when proper solvent was used. Chitosan, and its derivatives, unlike many of the other

The C-1 proton was not observable in most instances as it is known to appear near the signal due to water ($\delta \approx 4.6-4.9$) and the broad peak due to latter made it difficult to locate. Nevertheless, the information from the spectrum is quite sufficient to calculate the degree of deacetylation (D_{deac}) and the calculation was done as follows.

$$\begin{aligned} \text{Equation 2} \quad \% D_{\text{deac}} &= \left[1 - \frac{\left(\frac{1}{3} \times (\text{Signal intensity due to acetyl group}) \right)}{\left(\frac{1}{5} \times (\text{Signal intensity due to 3,4,5,6,6'}) \right)} \right] \times 100\% \\ &= \left[1 - \frac{5 \times (\text{Signal intensity due to acetyl group})}{3 \times (\text{Signal intensity due to 3,4,5,6,6'})} \right] \times 100\% \end{aligned}$$

The degree of deacetylation for the chitosan substrate was calculated to be 83.2% using Equation 2. The average MW of the repeating unit was determined based upon the degree of deacetylation as follows-

$$\begin{aligned} \text{Equation 3} \quad \text{Average MW of the repeating unit in chitosan} &= \\ &= \frac{[(\% D_{\text{deac}}) \times (\text{MW of single unit in chitosan})] + [(100 - \% D_{\text{deac}}) \times (\text{MW of single unit in chitin})]}{100} \\ &= \frac{[(83.18) \times (161.158)] + [(16.82) \times (203.194)]}{100} \\ &= 168.23 \end{aligned}$$

2.4 Experimental

2.4.1 Materials, Methods and Storage

Chitosan was purchased from Aldrich Chemical Co. and Fluka and came in three different MW types; Low MW ($\approx 150,000$), medium MW ($\approx 400,000$) and high MW (\approx

600,000). All other organic compounds were purchased from either Aldrich Chemical Co., Acros Organics or Lancaster Synthesis Company, except Quat-188, which was obtained from Dow Chemical Co. Solvents used were of HPLC grade and obtained mainly from Aldrich Chemical Co. and were used without further purification. All other chemicals including chitosan were used as such, in the form of purity they arrived. Semi-permeable membranes used for dialysis of the samples were Spectra/Por, manufactured by Spectrum Laboratories Inc. with a molecular weight cut-off (MWCO) of 6,000-8,000. pH Hydrion™ Insta-Check 0-13 test paper (Micro Essential Laboratory, NY) was used in estimating the pH. Products once recovered in pure and dry state were stable to air, moisture and room temperature.

Products obtained were mainly characterized using ^1H NMR spectroscopy. The spectra were recorded on a Bruker AC250 in D_2O (TSP as an internal standard) or 1% d_4 -acetic acid in D_2O as the solvent depending upon the solubility of analytes. Spectra were recorded at room temperature with 24 transients using a 90° flip angle and a repetition time of 10 sec. FTIR spectra were recorded on Bruker Tensor™ 27 instrument with a Pike MIRacle™ ATR (Attenuated Total Reflectance) detector.

2.4.2 Syntheses

Different hydrophobic derivatives of chitosan were prepared using one of the three methods, *viz.* Bosch reduction, γ -lactone addition or anhydride addition. Chitosan derivatives were synthesized with various substituents and at different levels of substitution for any given substituent.

2.4.2.1 Representative Procedure for Bosch Reduction

Chitosan (1g, 5.2 meq. of NH_2) was first dissolved in 1% (0.2M) acetic acid (100mL). To the above stirred solution was added glacial acetic acid (3mL) followed by the addition of benzaldehyde (0.132g, 0.2 meq.). The reaction mixture was further stirred at room temperature for 6-10 hours to ensure completion of Schiff base formation which was marked by change in the appearance of solution from being clear at the beginning but turning milky-white with the progress of reaction. NaBH_3CN (0.2 meq.) was now added to the reaction mixture in one portion, at room temperature and stirring continued for 12-14 hours, after which the milky appearance disappeared and a clear solution resulted. At this point the pH of the solution was adjusted to 7[†] using 15% aqueous NaOH and the resulting fibrous solid was filtered and washed several times with water. The damp solid was washed sequentially with four 100mL aliquots of acetone to produce a powdered product. After drying under nitrogen for 12 hours, 0.987g of material was obtained. When the complete isolation of the chitosan derivative thus prepared was not required, the fibrous solid obtained after washing with water was carried forward, while still moist, for the quaternization step using Quat-188.

2.4.2.2 Representative Procedure for γ -Lactone and Anhydride Addition

The procedure was similar to that in Procedure I above, except, the reaction time in the first step was 12-14 hours (instead of 6-10 hours) and the reduction step with NaBH_3CN was not carried out. After 12-14 hours of the initial step, a clear solution resulted which

[†] certain derivatives of chitosan, particularly those with acidic moieties present in the substituent remained dissolved at this pH. Under these circumstances, the pH of the medium was adjusted to a different value (usually greater than 7) such that precipitation of the product was complete.

was neutralized to pH 7[‡] using 15% aqueous NaOH and the resulting fibrous solid filtered and washed with water. The substituted chitosan thus obtained was either isolated by exhaustive acetone washing as described above or was carried forward for quaternization with Quat-188, while the solid was still moist.

2.4.2.3 Quaternization of Hydrophobic Chitosan Derivatives

Chitosan derivatives obtained as described above (starting with 0.5g chitosan), while still moist, were suspended and stirred for 20 min. in commercially available 65% w/w solution of Quat-188 (30mL). The reaction mixture at this point appeared as thick, uniformly dispersed slurry. A room temperature solution of NaOH (8.5g) dissolved in water (10mL) was poured into this slurry and stirring continued for next 18 hours (reaction mixture warmed up during the initial period following the addition of NaOH). In the later part of the project, a combination of Quat-188 (40mL) and room temperature solution of NaOH (5.7g) in water (13mL) was employed during the quaternization process, which produced identical results. Water (100mL) was now added to the thick solution thus obtained and the reaction mixture was heated to 60°C while stirring for 4-8 hour. This process usually dissolved any insoluble solids still present within the solution. Next, after cooling the reaction mixture to room temperature, the solution pH was adjusted to 7 using conc. HCl. The resulting substituted chitosan-quaternized derivative remained dissolved in the aqueous reaction medium and the reaction solution appeared colorless (in most cases) and clear. The product solution (\approx 200-250mL for a synthesis starting with approx. 0.5g of chitosan derivative) was dialyzed in water (\approx 4L) for 2 days.

[‡] see the footnote applicable to section 2.4.2.1.

The dialysis bath water was replaced every 12-14 hours. The resulting solution from the dialysis membrane was then either freeze-dried, or was first concentrated under vacuum (to about 30mL) on a rotary evaporator and product precipitated from the concentrated solution by adding acetone (\approx 600mL). The product was then filtered and further washed with acetone (\approx 400mL). The solid thus obtained was then collected and dried overnight at room temperature under a stream of nitrogen.

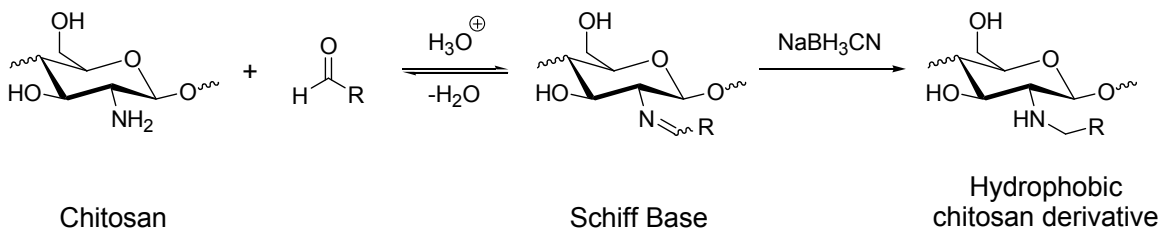
2.5 Results and Discussion

Previous work conducted within the Daly research group towards the syntheses of chitosan derivatives involved the use of hydrophilic, water soluble organic substrates.²⁷ The present work however required introducing hydrophobic substituents, the source of which in most cases would have to be from hydrophobic, water insoluble organic compounds. Thus, one of the first objectives of this work was to establish an efficient and versatile synthetic route towards the hydrophobic chitosan derivatives under heterogeneous reaction conditions. Once synthesized, these derivatives were further quaternized using Quat-188. It was realized from early on in this project that the key, synthetically demanding transformation would be that of introducing hydrophobic groups on the chitosan backbone and at the levels that could be controlled externally. Quaternization process using Quat-188 has been very well established during past several years in this laboratory and it worked out swiftly in this project just like in the past.

2.5.1 Bosch Reduction

Bosch reduction (Scheme 15) provides an attractive route for carrying out reductive amination of an amine with various organic aldehydes. Furthermore, given the fact that

chitosan is insoluble under neutral and basic conditions and the possibility of carrying out Bosch reduction under acidic conditions thereby attaining homogeneity of the process was thought to be highly favorable. It is worth noting that this synthetic route results into a secondary amine derivative of chitosan, *i.e.* with an alkyl substituent.



Scheme 15: Synthesis of hydrophobic chitosan derivatives- Bosch reduction

Reductive amination of chitosan (Bosch reduction, Scheme 15) is a very attractive synthetic route since it provides with the versatility of introducing an array of substituents. We employed various aldehydes, at different mole ratios with respect to the amine of chitosan, in order to vary the structural patterns and the degree of hydrophobicity along the macromolecular chain. The reaction was carried out under heterogeneous conditions where the organic aldehyde remained dispersed in the solution of chitosan in aqueous 1% acetic acid. There are two distinct steps involved in this process; first the formation of Schiff base and the second, reduction of the Schiff base to an amine. A change in the appearance of the reaction mixture occurred as the reaction progressed during the Schiff base formation. Thus, a clear solution of chitosan gradually turned turbid with time after the addition of the aldehyde and eventually became opaque, milky-white in appearance. With the help of control experiments performed in absence of any chitosan, we ruled out the possibility that the turbidity of the solution resulted from an emulsion formed by the water-insoluble organic aldehyde in the aqueous reaction

medium. Additionally the turbidity did not seem to result from any precipitation taking place within the reaction mixture, as no solid could be isolated from the turbid suspension by means of filtration. It was therefore concluded that the appearance of turbidity was directly related to the progress of reaction and increasing turbidity was an indication of it. When the turbid suspension that resulted from the treatment of chitosan with *n*-octyl aldehyde for 6-10 hours was neutralized to pH 7, a fibrous solid resulted. ^1H NMR analysis of this solid (Figure 10) showed the presence of imine characterized by the imine proton signal ($\delta \approx 8.3$). However, the strength of this signal was weak compared to the anticipated value. Based on these observations, it appears that Schiff base formation with chitosan is a rapid, reversible process that is highly dependant upon the pH of the reaction mixture.

In the second step, the Schiff base was reduced to the corresponding substituted chitosan derivative using NaBH_3CN as the reducing agent. The turbidity formed as a result of Schiff base formation disappeared upon the reduction and the solution turned clear. It was also noted that the reductive amination route worked best when aromatic aldehydes were employed. Aliphatic aldehydes generally resulted in poor substitution, unless a co-solvent was incorporated during the synthesis. The results obtained are described below.

2.5.1.1 Hydrophobic Substitution Using Aromatic Aldehydes

Different aromatic aldehydes were employed in the Bosch reduction methodology in this project. Possibility of external control on the degree of substitution was studied by varying the mole proportions of the aldehyde with respect to the amine of chitosan. In order to establish the extent of substitution and hence calculate the % recovery yield of

the polymer, it was necessary to determine the degree of deacetylation of the chitosan sample used. The latter was established using ^1H NMR method as described in section 2.3. The aromatic substituents could be advantageous in the process of establishing the degree of substitution using the ^1H NMR method. Aromatic proton resonances appear in the downfield region compared to the residual sugar protons and therefore they can be integrated with minimal interference leading to greater accuracy. A representative spectrum of the substituted chitosan with aromatic substituent (benzyl) is given above (Figure 5).

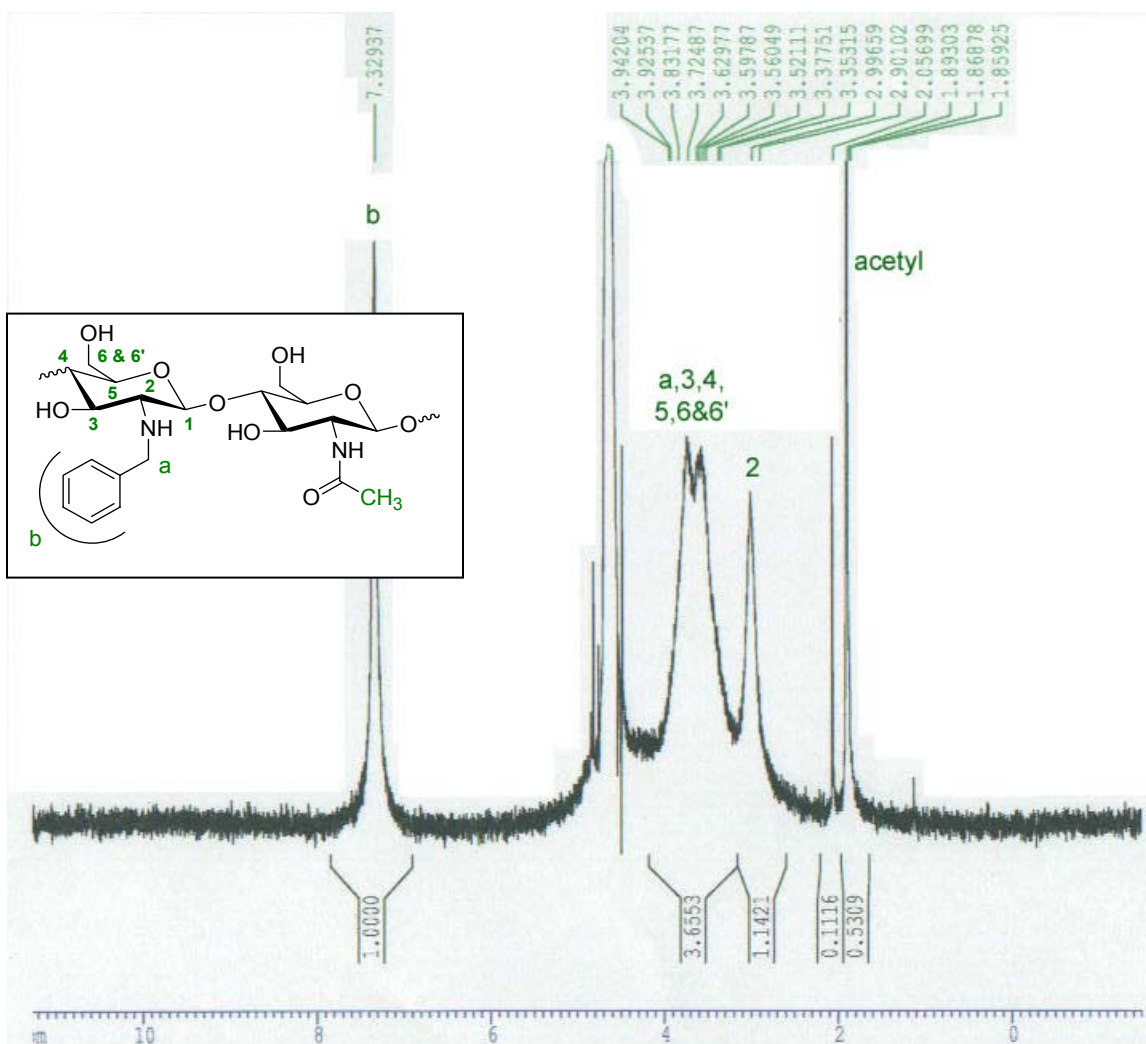


Figure 5: ^1H NMR spectrum of benzyl chitosan in 1% d_4 -acetic acid in D_2O

The substitution reaction involved using 0.2 equivalents (20 mol%) of benzaldehyde with respect to the amine of chitosan. The following approach was used in order to calculate the degree of substitution. ^1H NMR spectrum was used to compare the intensity of the C-2 proton signal with that of the aromatic protons. The following relationship applies to the spectrum shown in Figure 5.

$$\frac{(\text{Signal intensity due to 2})}{(\text{Signal intensity due to b})} = \frac{(\text{Number of C - 2 protons per sugar residue})}{(\text{Number of aromatic protons per sugar residue})}$$

Assuming the degree of substitution to be Y%, we get

$$\frac{(\text{Signal intensity due to 2})}{(\text{Signal intensity due to b})} = \frac{1_{(c-2)}}{\left(\frac{5_{(arom.)} \times Y}{100} \right)}$$

Rearranging the terms, we get

Equation 4

$$Y = \left(\frac{1_{(c-2)} \times (\text{Signal intensity due to b})}{5_{(arom.)} \times (\text{Signal intensity due to 2})} \right) \times 100\%$$

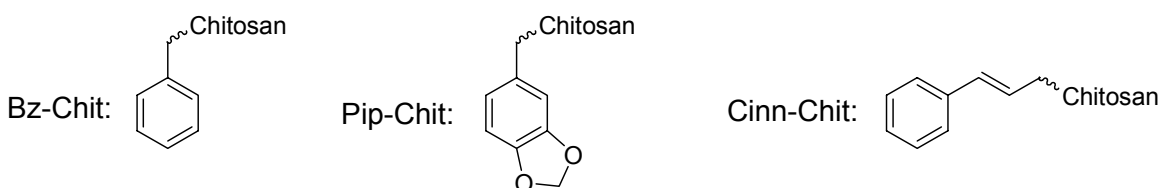
Using the approach above (Equation 4), the % substitution was calculated to be 17.5% for the benzyl derivative (Figure 5). Equations 4 can generally be applied to any of the chitosan derivatives by making appropriate adjustments to the coefficients used as dictated by the structure of the substituent. For example, if the aromatic substituent contained 4 aromatic protons instead of 5, the term $5_{(arom.)}$ in Equation 4 should be replaced with $4_{(arom.)}$; etc.

The different aromatic aldehydes employed in derivatizing chitosan are listed below (Tables 3, 4, 5, 6) along with the % substitution and % recovery of the polymer products obtained. It is worth mentioning here that one of the most important aspects of this study

was to establish whether or not it was possible to have control on % substitution by external means.

Table 3: Chitosan derivatives with aromatic substituents

Entry	Derivative	% substitution targeted	% substitution obtained	% recovery
1	Bz-Chit	20.00	17.5	90.2
2	Pip-Chit	34.95	12.4	75.7
3	Cinn-Chit	35.68	22.6	74.8



Bz-Chit, Pip-Chit and Cinn-Chit derivatives were prepared from benzaldehyde, piperonal and *trans*-cinnamaldehyde respectively. Reasonable substitution control was realized for the benzaldehyde derivative (Table 3; Entry 1), whereas piperonal and *trans*-cinnamaldehyde provided lower than anticipated levels of substitution under similar reaction conditions (Table 3; Entries 2 and 3). The lower levels of % substitution in the latter two cases are probably due to the differences in the reactivities of these aldehydes.

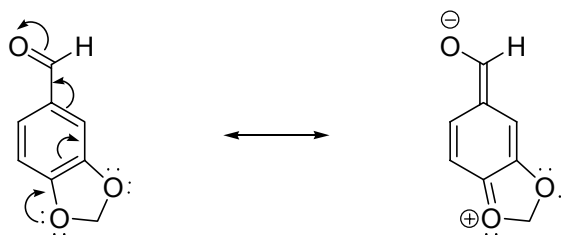


Figure 6: Diminished electrophilicity of the carbonyl group in piperonal

In the case of piperonal this difference is more evident. The carbonyl group in piperonal is rendered less electrophilic as a result of the mesomeric effect of the electron rich acetal oxygen at the *para* position (Figure 6). This probably directly affects the Schiff base

formation equilibrium (Scheme 15) and results into lower substitution levels. Table 4 provides the % substitution results in the case of aromatic aldehydes bearing sulfonic acid moieties. Entries 1 thru 4 involved reaction of chitosan with 2-formyl-benzene sulfonic acid, sodium salt while entries 5 and 6 used 6-formyl-1,3-benzene disulfonic acid, bissodium salt. It can be seen from Table 4 that, electron withdrawing sulfonic acid group provides good % substitution that can be well controlled externally (Figure 7). A logarithmic relationship is observed between % substitutions obtained and % substitutions targeted. This strongly suggests that desired levels of substitution are attainable by varying the mole ratios of chitosan and 2-formyl-benzene sulfonic acid, sodium salt, thereby providing means of external control.

Table 4: Chitosan derivatives with aromatic sulfonic acid substituent

Entry	Derivative	% substitution targeted	% substitution calculated	% recovery
1	2-sulf-Bz-Chit	10.0	8.2	99.9
2	2-sulf-Bz-Chit	20.0	16.9	86.4
3	2-sulf-Bz-Chit	30.0	22.1	74.7
4	2-sulf-Bz-Chit	50.0	30.4	63.4
5	2,4-disulf-Bz-Chit	10.0	5.7	84.2
6	2,4-disulf-Bz-Chit	20.0	12.2	90.7

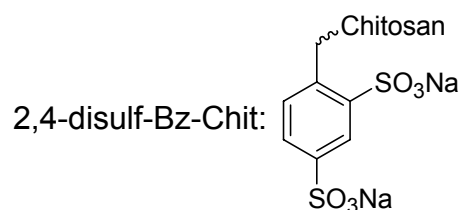
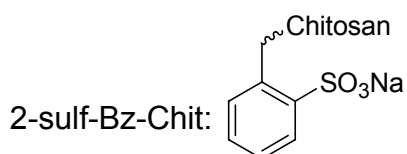


Table 5 and Table 6 provide results obtained for 4-carbox-Bz-Chit and 4-acetox-Bz-Chit derivatives respectively. These derivatives were prepared from 4-carboxy benzaldehyde and 4-acetoxy benzaldehyde respectively. Substitution trends observed

(Figure 8 and Figure 9 respectively) are similar to those observed with 2-formyl-benzene sulfonic acid, sodium salt discussed earlier (Figure 7).

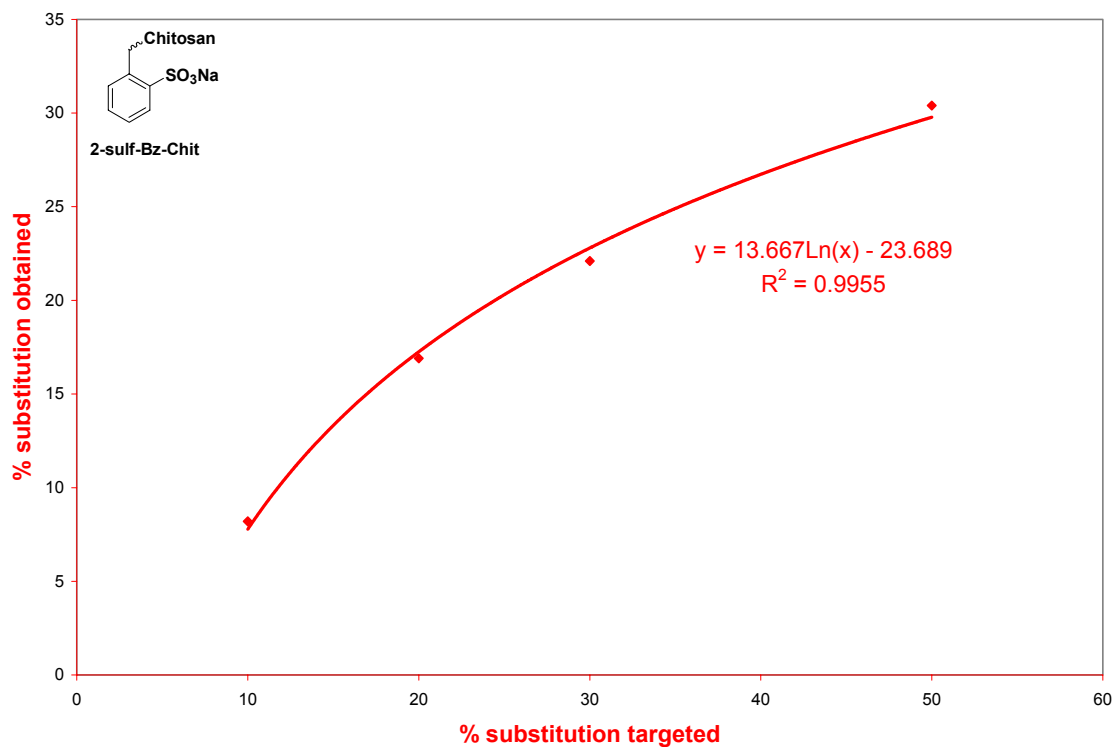
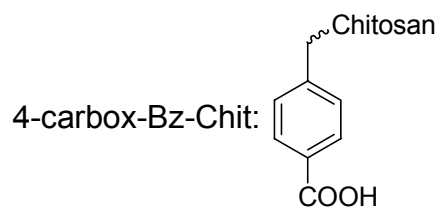


Figure 7: Substitution control in the synthesis of 2-sulf-Bz-Chit

Table 5: Chitosan derivatives with aromatic carboxy substituent

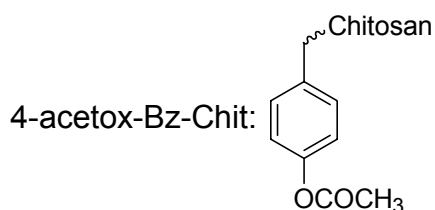
Entry	Derivative	% substitution targeted	% substitution calculated	% recovery
1	4-carbox-Bz-Chit	10.00	8.2	22.2
2	4-carbox-Bz-Chit	20.00	15.6	77.4
3	4-carbox-Bz-Chit	30.00	20.3	76.4



Chitosan derivative 4-carbox-Bz-Chit (Table 5) showed, in general, a lower % recovery compared to the sulfonic acid derivatives (Table 4). This is due to the difficulties during the isolation process for these derivatives in which a suitable pH was rather difficult to attain at which complete precipitation of product occurred. It is however possible under these circumstances to improve the % recovery by carrying out dialysis of the product solution followed by freeze-drying or acetone precipitation. In addition to the acidic derivatives described earlier, a hydrophobic neutral aromatic derivative of chitosan, 4-acetox-Bz-Chit demonstrated good control of substitution (Table 6 and Figure 9).

Table 6: Chitosan derivatives with aromatic acetoxy substituent

Entry	Derivative	% substitution. targeted	% substitution calculated	% recovery
1	4-acetox-Bz-Chit	10.0	6.3	14.2
2	4-acetox-Bz-Chit	20.0	13.7	83.5
3	4-acetox-Bz-Chit	30.0	19.9	91.9



However, comparing Table 6 with Table 5, it is clear that the absolute values of % substitution calculated for 4-acetox-Bz-Chit are lower than that of 4-carbox-Bz-Chit at same levels of targeted % substitutions. This observation suggests a possible Hammett type relationship that might exist for Bosch reduction methodology using chitosan in the preparation of 2-sulf-Bz-Chit, 4-carbox-Bz-Chit and 4-acetox-Bz-Chit derivatives. It is however difficult to obtain reliable *ortho* substituent coefficients (σ_o) from literature, as

would be required in the case of 2-sulf-Bz-Chit; which are necessary in exploring any free energy correlation. And therefore, with the available data, such a relationship could not be pursued with confidence.

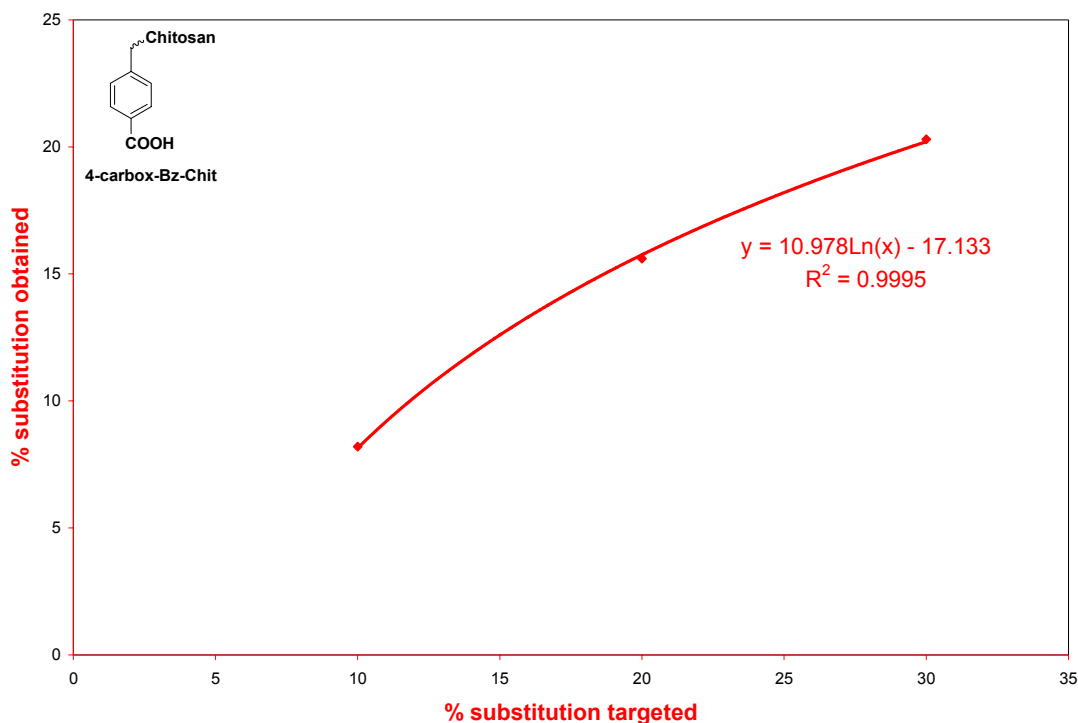


Figure 8: Substitution control in the synthesis of 4-carbox-Bz-Chit

In summary, it can be concluded that aromatic substitution following Bosch reduction approach is a highly feasible synthetic protocol for obtaining substituted chitosans. Further, the results obtained here suggest that the levels of substitution can be very well controlled externally, by varying molar ratios of the aromatic aldehyde and chitosan. This finding is remarkable especially considering that Bosch reduction methodology was performed under heterogeneous reaction conditions. Additionally, the % substitution obtained during Bosch reduction was found to be dependent on the nature of substituent on the aromatic aldehyde. This study also suggested that ^1H NMR can be used as a tool

to establish % substitution, so long as complete dissolution of the chitosan derivative is achieved in an appropriate solvent.

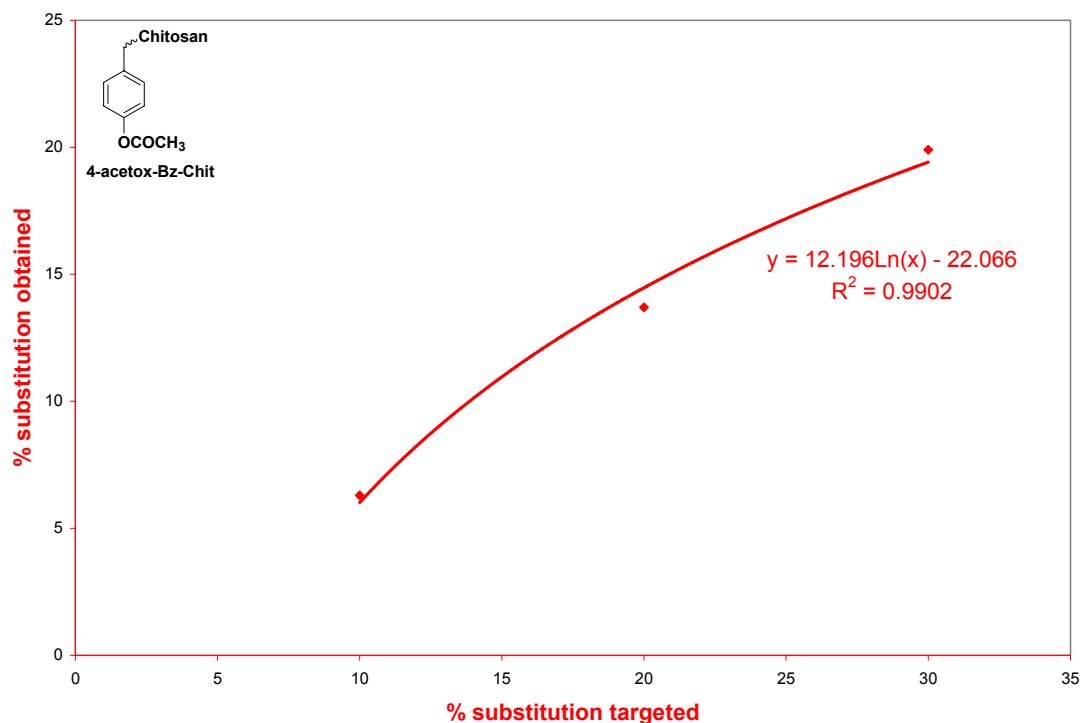


Figure 9: Substitution control in the synthesis of 4-acetox-Bz-Chit

2.5.1.2 Hydrophobic Substitution Using Aliphatic Aldehydes

Aliphatic aldehyde used in derivatizing chitosan was mainly *n*-octyl aldehyde, a hydrophobic water-immiscible aldehyde. Additionally, certain chitosan gels were prepared using water-soluble glutaraldehyde and results obtained in chitosan gel studies are discussed in Chapter 4 (section 4.3, *vide infra*).

Initial attempts towards isolating the Schiff base formed by the reaction of chitosan and *n*-octyl aldehyde proved futile, since during the process of isolation the pH change in the reaction mixture caused major hydrolysis of this intermediate. It was also observed that once isolated, the Schiff base further degraded in contact with moisture from the air, the extent of this degradation was not determined. It would have been of advantage from

a synthetic perspective to be able to isolate the imine obtained from the reaction of chitosan with various aldehydes; as this opens up the possibility to carry out different synthetic transformations that are typical of imine, thereby providing more than one means for hydrophobic substitution.

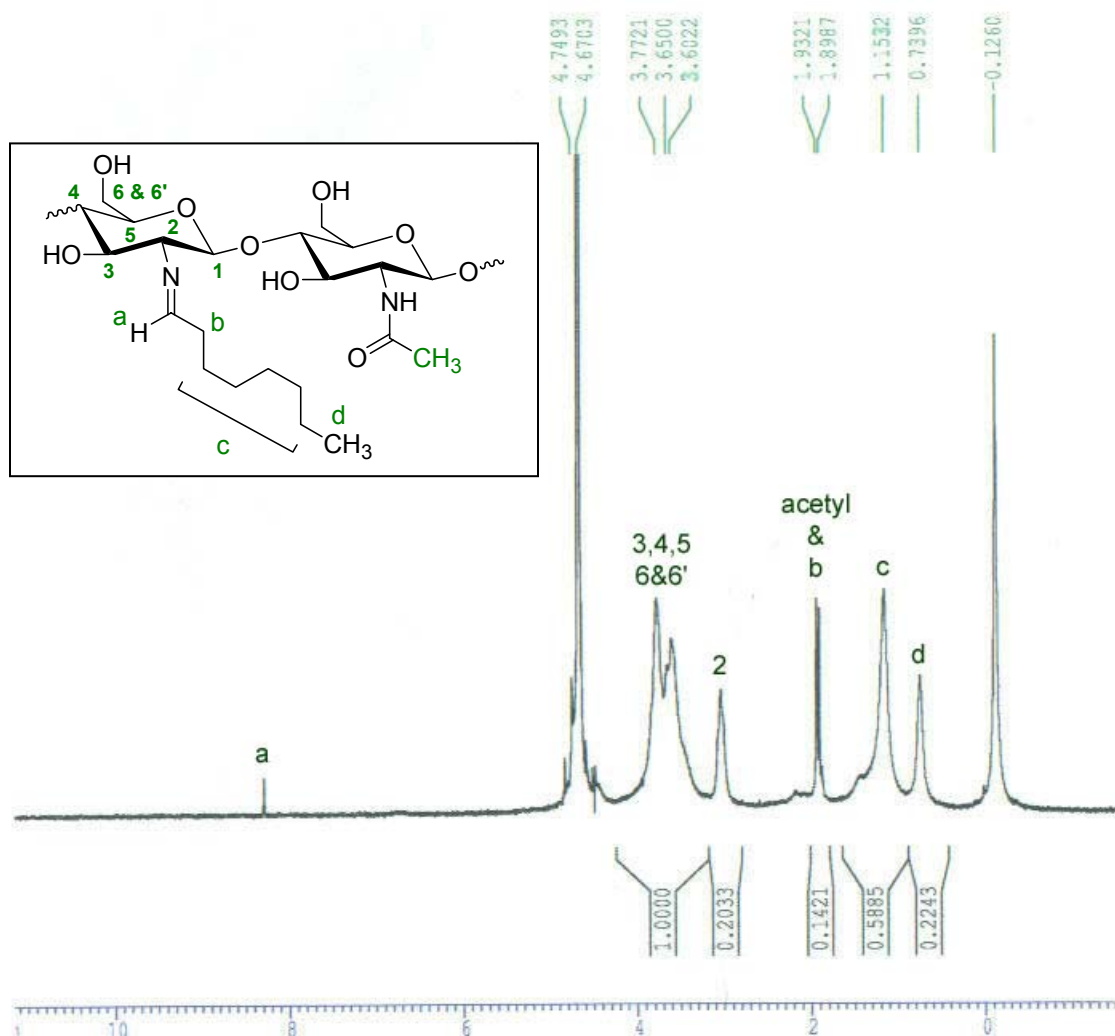


Figure 10: ¹H NMR of the Schiff base obtained from *n*-octyl aldehyde and chitosan in 1% *d*-4 acetic acid in D₂O

It was however possible to obtain the ¹H NMR of the intermediate Schiff base (Figure 10), but with much less signal intensity due to imine proton than anticipated. Schiff base isolation attempts were hence abandoned and instead *in situ* reduction approach was

followed in this project. % substitution calculations for hydrophobic aliphatic derivatives were performed using Equation 4 with appropriate modifications. % substitution for the imine represented in Figure 10 was calculated to be 36.8% by comparing the methyl signal from the octyl side-chain (d) with the C-2 proton of chitosan. A preliminary study evaluating the best possible reaction conditions for Bosch reduction methodology was performed using *n*-octyl aldehyde. The reaction was found more facile at room temperature compared to at higher temperatures, e.g. at 60°C. The results from the optimization studies performed are discussed in Chapter 3 in more detail (*vide infra*).

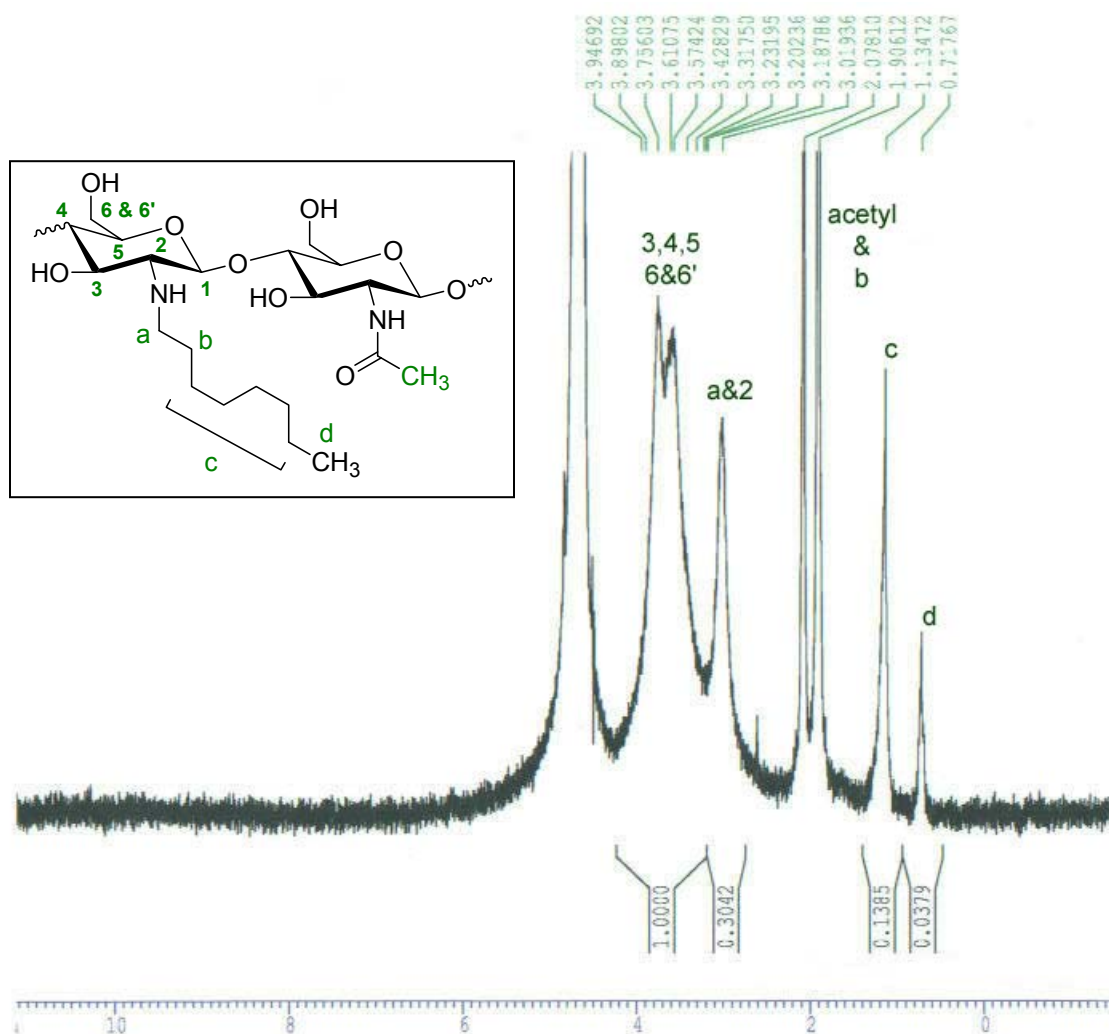
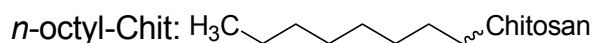


Figure 11: ¹H NMR of *n*-octyl chitosan in 1% *d*-4 acetic acid in D₂O

¹H NMR spectrum of *n*-octyl chitosan is given in Figure 11. The % substitution in this derivative was determined by comparing the methyl signal intensity (d) with that of the sugar protons 3, 4, 5, 6 and 6' (using to Equation 4 with appropriate modifications). The % substitution thus obtained was 4.2%. Table 7 provides the results obtained under Bosch methodology using *n*-octyl aldehyde.

Table 7: Bosch reduction using *n*-octyl aldehyde

Entry	Derivative	% substitution. targeted	% substitution calculated	% recovery
1	<i>n</i> -octyl-Chit	20.0	4.2	74.9
2	<i>n</i> -octyl-Chit	35.0	5.5	67.0
3	<i>n</i> -octyl-Chit	100.0	5.8	83.5
4	<i>n</i> -octyl-Chit	200.0	4.8	91.9



The results obtained using representative aliphatic aldehyde did not show any trend between the targeted % substitution and % substitution obtained. In addition, the levels of substitution were substantially low. This sharp contrast between aliphatic and aromatic aldehydes could possibly be due to the differences in their reactivities. It was however possible to improve the % substitution and attain external control of substitution in reactions employing aliphatic aldehydes by using an organic co-solvent (*viz.* DMF) during the course of reaction (section 3.5, *vide infra*).

In summary, hydrophobic substitution using long-chain water-immiscible aliphatic aldehyde in a Bosch reduction methodology resulted in lower % substitutions. Attempts toward isolation of the Schiff base intermediate were hampered by the fact that the intermediate was unstable and underwent degradation during the isolation process. Upon careful isolation, the Schiff base intermediate was found to be unstable to air and

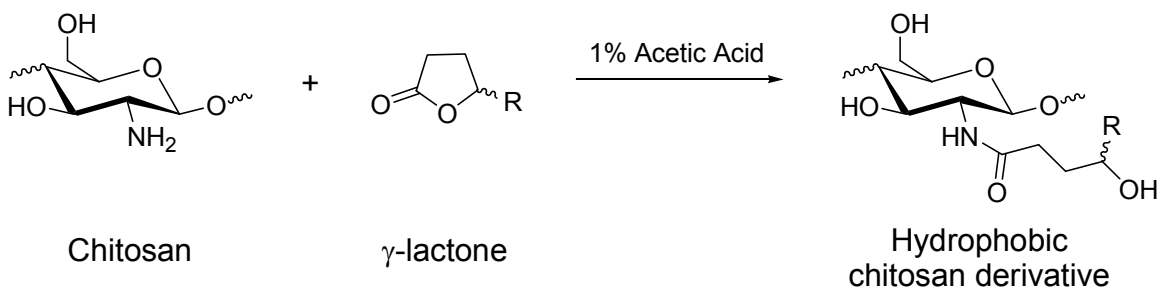
moisture as was evidenced by the release of the aldehyde from the material. The % substitutions obtained under Bosch reduction methodology showed a sharp contrast between water-soluble aldehydes and water-immiscible aldehydes used during the process.

2.5.2 γ -Lactone Addition and Anhydride Addition

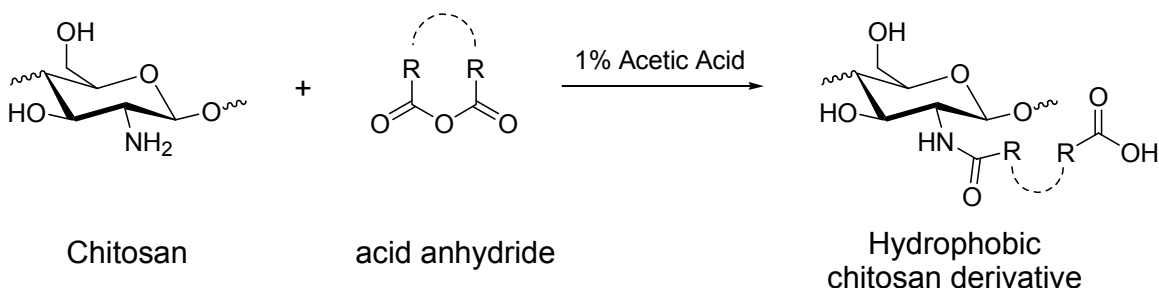
In addition to Bosch reduction, hydrophobic derivatives of chitosan were also synthesized using γ -lactone addition and anhydride addition routes (Scheme 16 and Scheme 17 respectively). Unlike Bosch reduction, these two routes incorporate hydrophobic residues through carboxamide connectivity. In addition, γ -lactone addition generates a δ -hydroxy group in the sidechain with respect to the nitrogen of chitosan. Previous research in the Daly research group has shown derivatives of chitosan with β -hydroxy group on the substituent with respect to the nitrogen of chitosan to exhibit excellent antibacterial activity.²⁷ However, the activity was adversely affected upon derivatization of this hydroxy group to an ester.²⁷ It would thus be worthwhile to look for any effect due to a δ -hydroxy derivative formed under γ -lactone addition route on the antibacterial activity. An anhydride addition route generates a carboxamide linkage of the substituent and may provide an ω -carboxylic acid substituent, provided that the anhydride used is a cyclic (internal) anhydride. The reaction conditions used are mildly acidic (1% acetic acid).

Decomposition of the macromolecule during the course of syntheses, thereby resulting in a product with reduced degree of polymerization (DP) under these reaction conditions, is shown in the literature to be not an issue of concern.⁵⁸ Among the three synthetic

routes given above, the latter two approaches clearly have an advantage over Bosch reduction as ‘scale-up’ friendly routes. Our aim however was to study the effects of connectivity and the presence of hydroxy group in the substituent on the antibacterial activity of the resultant derivative.



Scheme 16: Synthesis of hydrophobic chitosan derivatives- γ -lactone addition



Scheme 17: Synthesis of hydrophobic chitosan derivatives- anhydride addition

γ -Lactone addition and anhydride addition reactions (Scheme 16 and Scheme 17 respectively) with chitosan were performed under heterogeneous reaction conditions, except in the case of γ -butyrolactone which is a water-miscible liquid. The choice of this methodology is attractive from the perspective of large scale synthesis and therefore possible industrial adaptability, mainly because the process does not require use of potentially hazardous NaBH_3CN as the reducing agent. This also makes the synthesis one step shorter. The process exploits the fact that primary nitrogen of amine in chitosan

is more nucleophilic than the hydroxyls and therefore selective *N*-acylation is possible to attain. The results obtained from these reactions are discussed below.

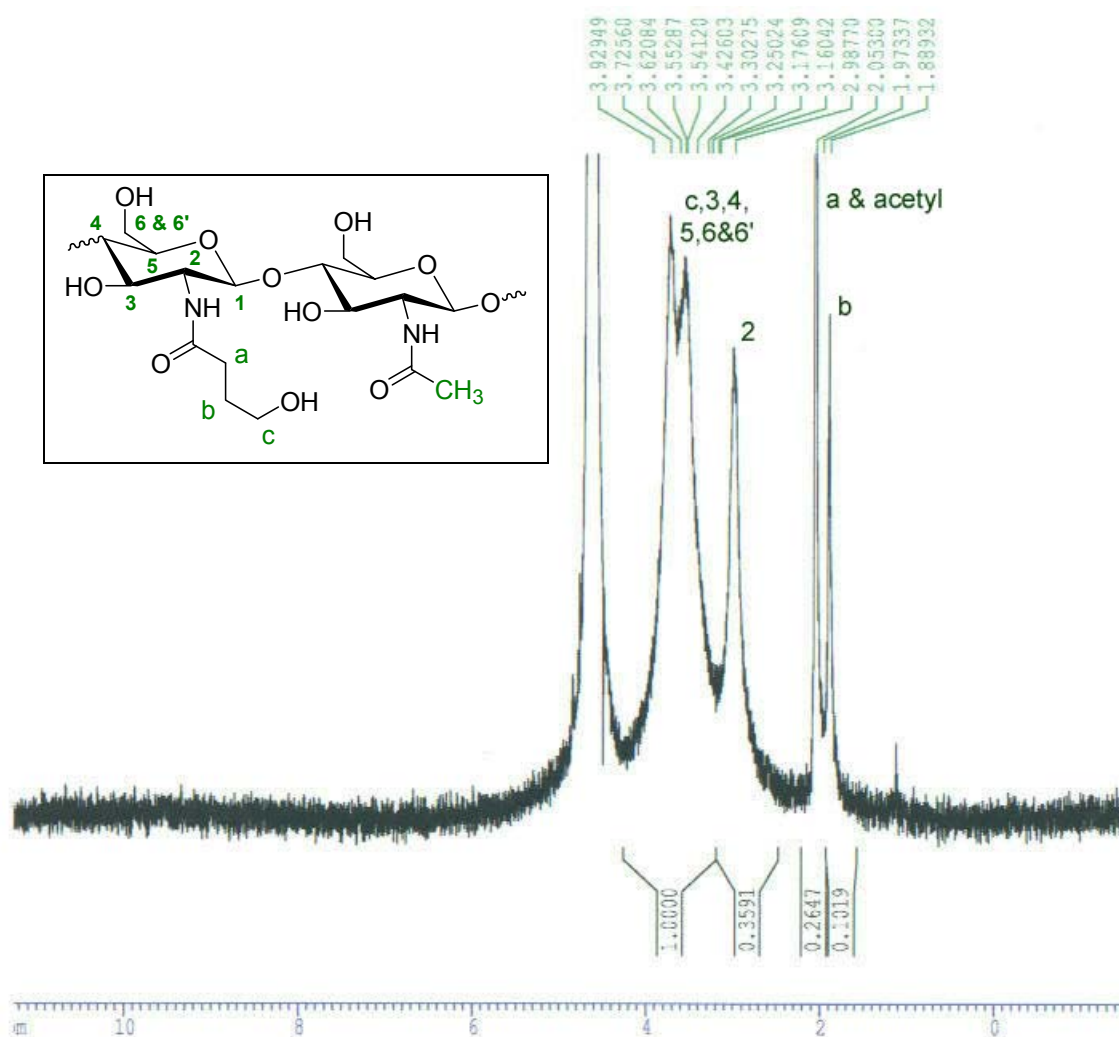


Figure 12: ^1H NMR of chitosan- γ -butyrolactone addition product in 1% *d*-4 acetic acid in D_2O

When γ -butyrolactone was treated with chitosan in a homogeneous process, the resultant addition product formed as expected (Figure 12). The % substitution was calculated using the signal intensities obtained from the ^1H NMR. The equation that is applicable for the ^1H NMR spectrum in Figure 12 is as follows-

$$\frac{(\text{Signal intensity due to c,3,4,5,6 \& 6'})}{(\text{Signal intensity due to b})} = \frac{(\text{Number of "c" hydrogens and sugar protons per sugar residue})}{(\text{Number of "b" protons per sugar residue})}$$

Assuming the degree of substitution to be Y%, we have

$$\frac{(\text{Signal intensity due to c,3,4,5,6 \& 6'})}{(\text{Signal intensity due to b})} = \frac{\left(\frac{2_{(Hs \alpha to -OH)} \times Y}{100} \right) + 5_{(sugar)}}{\frac{2_{(b)} \times Y}{100}}$$

Rearranging the terms, we get

$$\text{Equation 5} \quad Y = \frac{5_{(sugar)} \times 100}{\left[2_{(b)} \times \left(\frac{\text{Signal intensity due to c,3,4,5,6 \& 6'}}{\text{Signal intensity due to b}} \right) \right] - 2_{(H's \alpha to -OH)}} \%$$

Using Equation 5, % substitution was determined to be 28.4%. The % recovery of the substituted product was calculated based on % substitution and was found to be 86.2%.

A more hydrophobic derivative of chitosan using the γ -lactone addition methodology was envisioned in the substitution reaction using γ -octanoic lactone (4-butyl-dihydro-2(3*H*)-furanone). The water-immiscibility of this lactone resulted in heterogeneous reaction conditions. The products obtained with targeted % substitutions of 10%, 20% and 30% using this lactone showed the absence of any anticipated substitution resonances in the ^1H NMR spectra due to the aliphatic hydrocarbon chain. However, the FTIR analyses of the products revealed a prominent absorption bands at 1581cm^{-1} (N-H bend) and 1644cm^{-1} (C=O stretch), that were more intense than those in the native chitosan (compare Figure 13 and Figure 14). The products of the reaction with 10% and 30% targeted substitution showed a weight gain of 5.8% and 11.0% respectively.

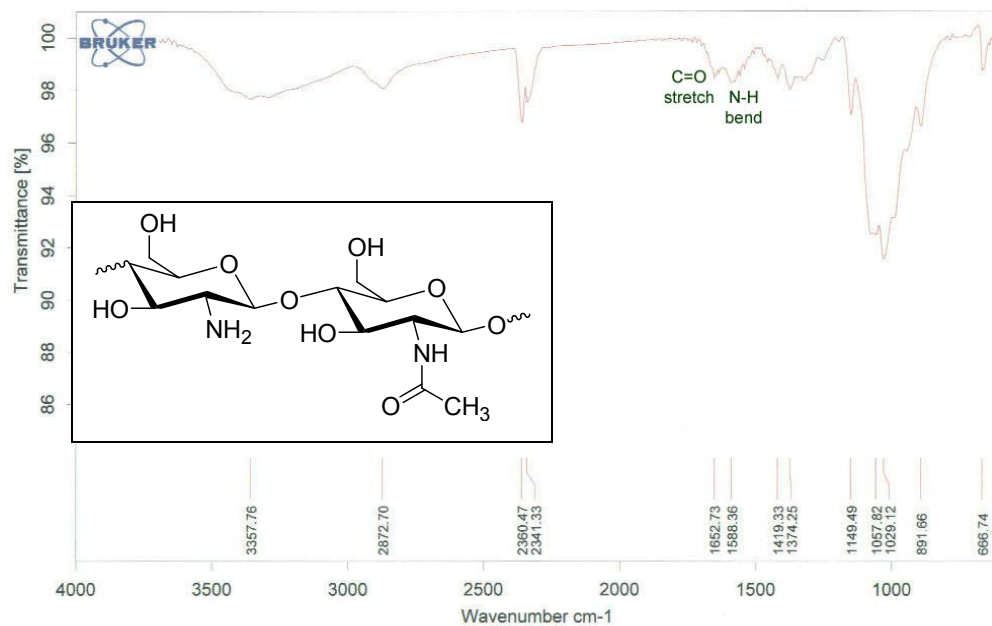


Figure 13: FTIR spectrum of chitosan (neat)

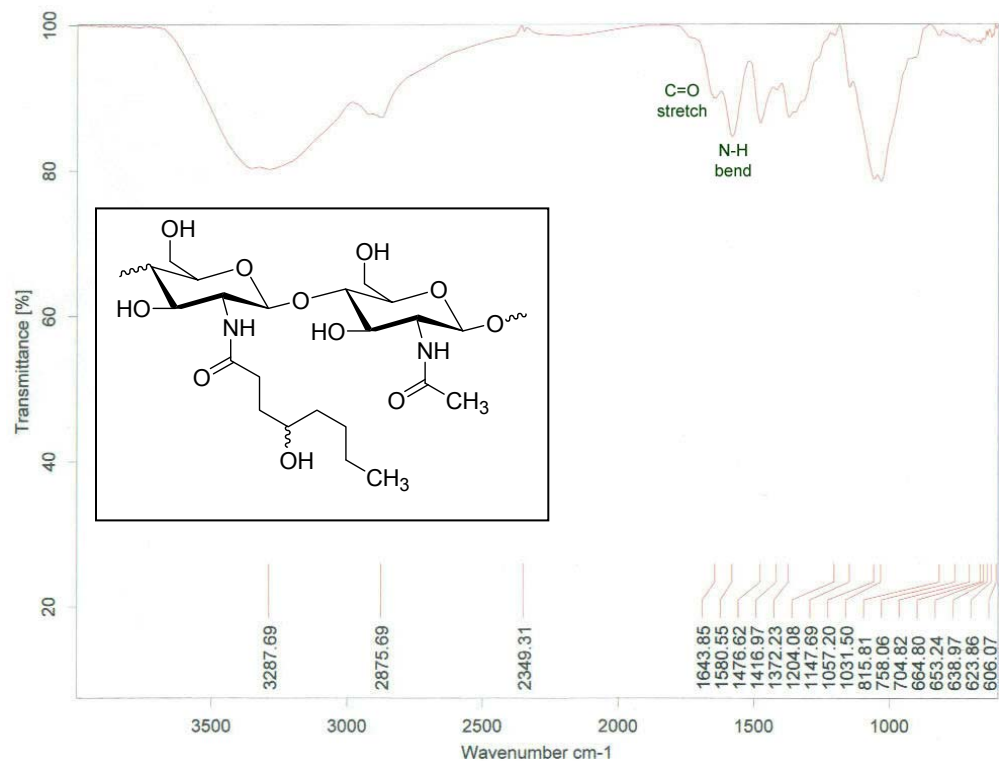
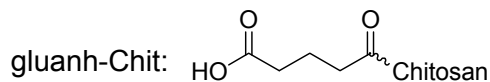
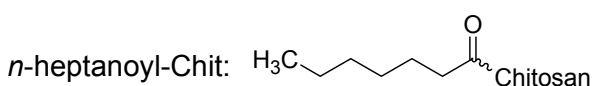


Figure 14: FTIR spectrum of substitution product from chitosan and γ -octanoic lactone (neat)

In addition, the appearances of products obtained after the reaction with γ -octanoic lactone were different from chitosan. Chitosan appeared as a flaky white solid, where as the products that resulted from these reactions were amorphous, off-white powders. Further, the materials obtained by reaction of chitosan with γ -octanoic lactone, upon quaternization exhibited marked difference in antibacterial activity against *E. coli* and *S. aureus* compared to that of Chit-Quat. These observations collectively suggest that substitution reactions of chitosan with γ -octanoic lactone under heterogeneous reaction conditions resulted in very low levels of substitution that could only be detected using FTIR. Figure 13 and Figure 14 exhibited a broad signal in the region 3600-3000 cm^{-1} . These signals are assigned as due to a combination of two stretches (O-H and N-H). These characteristic stretches are known to appear as broad signals and hence they can not be usually resolved into individual signals.

Table 8: Anhydride additions to chitosan

Entry	Derivative	% substitution. targeted	% substitution calculated	% recovery
1	<i>n</i> -heptanoyl-Chit	10.0	1.8	87.3
2	<i>n</i> -heptanoyl-Chit	20.0	2.4	83.1
3	<i>n</i> -heptanoyl-Chit	30.0	1.5	81.4
4	gluanh-Chit	35.0	10.1	86.2
5	gluanh-Chit	35.0	10.6	82.7



Anhydride substitution of chitosan was carried out using *n*-heptanoic anhydride and glutaric anhydride at different levels of targeted substitution. The results obtained are given in Table 8. Anhydride addition with hydrophobic, water-insoluble *n*-heptanoic

anhydride resulted in % substitutions much lower than targeted values. Further, no trend could be noted between substitutions targeted and obtained (Table 8; Entries 1-3). ^1H NMR spectrum of heptanoyl chitosan is shown in Figure 15.

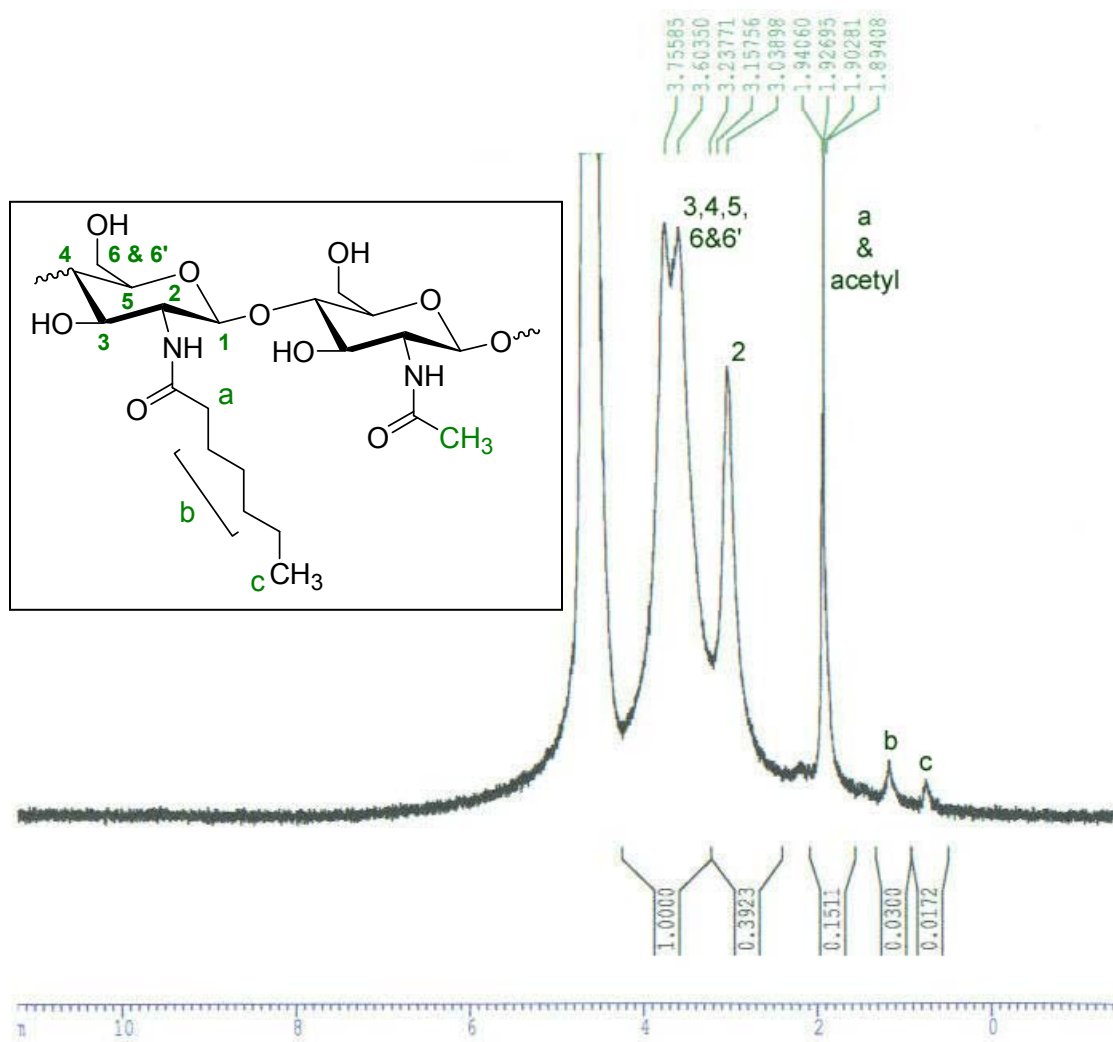


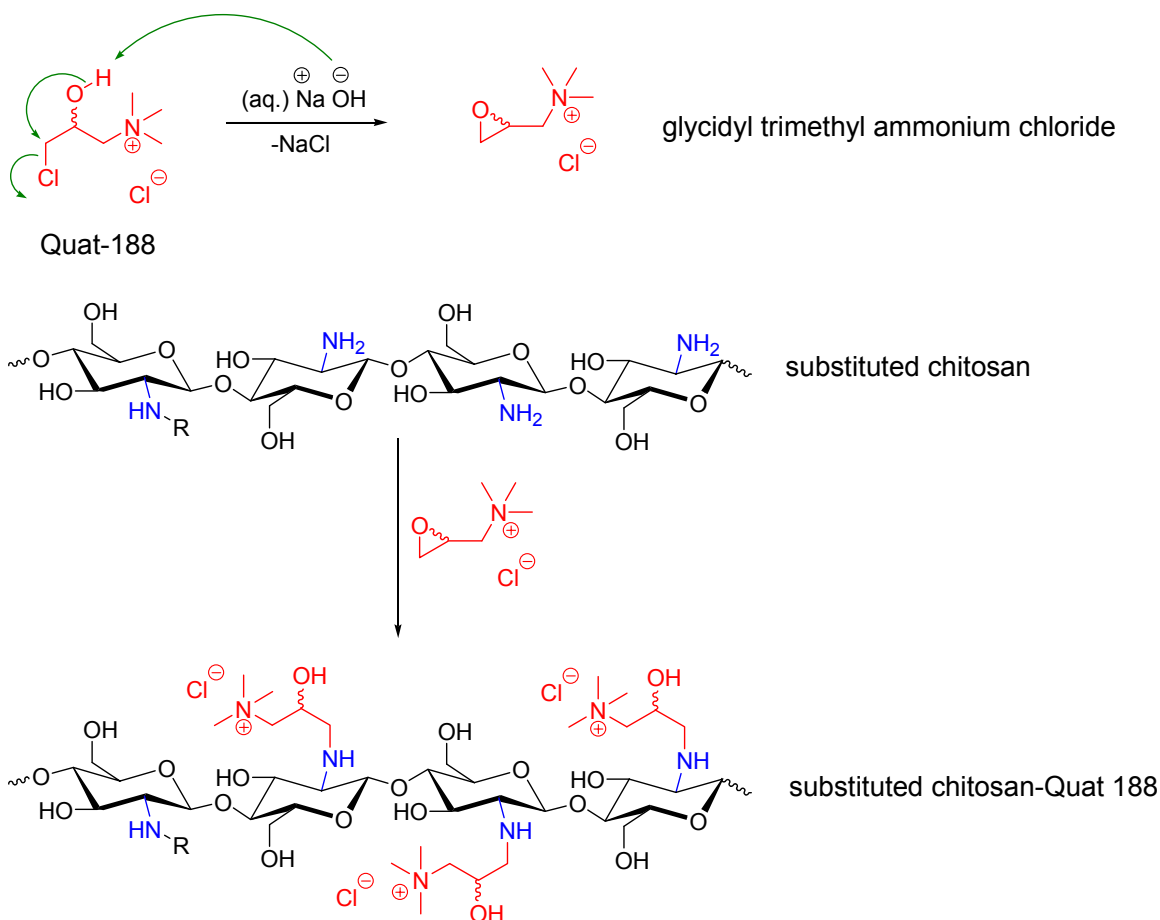
Figure 15: ^1H NMR spectrum of heptanoyl-chitosan in 1% *d*-4 acetic acid in D_2O

Glutaric anhydride showed better substitution control that was fairly reproducible with in the limits of experimental errors (Table 8; Entries 4 and 5). The marked differences in the reactivities of these two anhydrides are possibly due to the structural differences. Glutaric anhydride, a 6-membered cyclic anhydride undergoes a ring opening during the

addition reaction. Consequently, the release in ring strain is likely to be the reason for higher reactivity exhibited by glutaric anhydride.

2.5.3 Quaternization of Hydrophobic Chitosan Derivatives

Quaternization was performed using commercially available Quat-188 in a heterogeneous process under alkaline conditions. Under alkaline conditions, Quat-188 readily generates the corresponding epoxide, which reacts with the free primary amino groups of chitosan in a nucleophilic addition pathway (Scheme 18) resulting into quaternization. Quat-188 thus provides an effective alternative to the unstable epoxide itself while reducing possible hazards associated with using epoxides.



Scheme 18: Quaternization of hydrophobic chitosan derivatives

Quaternization process imparted fast and efficient water solubility to the hydrophobic chitosan derivatives at high concentrations and over a wide pH range. The process of quaternization using glycidyl trimethyl ammonium chloride has been reported to provide a degree of substitution equal to one.⁷¹ This means, under this reaction protocol, only the primary amino groups selectively react with the epoxide producing the quaternized derivative. Amines that are already quaternized even though capable, in principle, of a subsequent second quaternization, do not undergo such a transformation. Synthesizing water soluble derivative at neutral pH is essential to broaden the scope of commercial applications in consumer products.

Hydrophobic chitosan derivatives obtained from the syntheses described earlier were insoluble under neutral aqueous conditions. The soluble quaternized derivatives of these macromolecules were prepared by reaction with Quat-188 in presence of aqueous NaOH. It proved necessary to use a large excess of Quat-188 in this methodology as high % substitution with the quaternary ammonium group is only possible under such conditions.⁷¹ Attempts using Quat-188 on a mole-mole ratio proved futile. The material obtained in such cases remained under-quaternized, degree of which was not established, and as a result it was insoluble at pH 7. A representative ¹H NMR spectrum of a hydrophobic chitosan quaternized derivative is given in Figure 16. The spectrum in Figure 16 is the quaternized derivative of heptanoyl-chitosan represented in Figure 15.⁷² A strong signal due to acetyl and hydrogens α to the carbonyl group in the heptanoyl moiety ($\delta = 1.9$) supports the stability of amide groups during the quaternization process. Tables 9-13 provide different quaternized derivatives prepared in the current project. The degree of quaternization obtained using Quat-188 has been studied in detail in the Daly

group in past. Other reports from literature also support these findings that, under the reaction conditions employed, quaternization occurs at primary amino group sites only and with a high degree of quaternization (>0.9).⁷¹

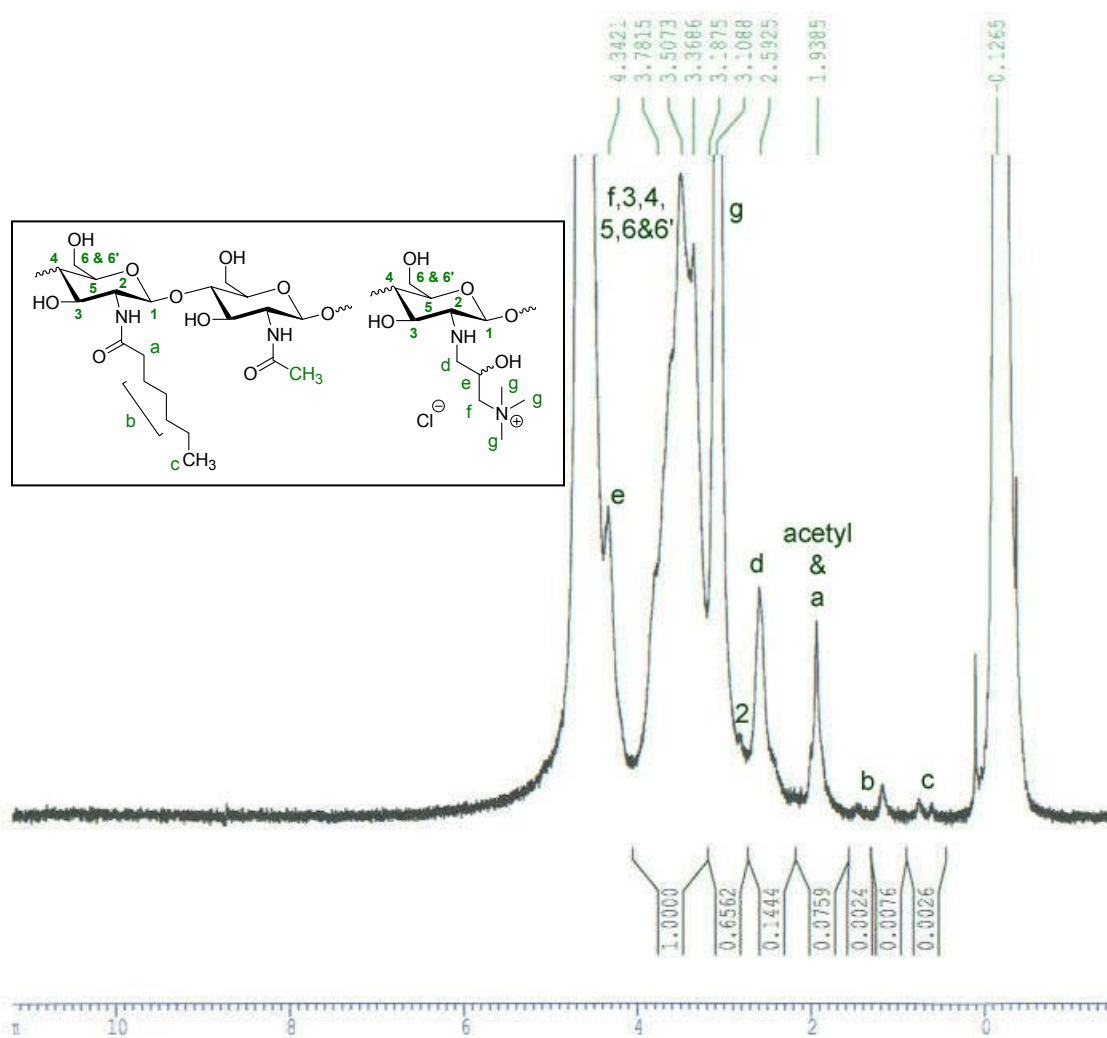


Figure 16: ^1H NMR spectrum of the quaternized derivative of heptanoyl-chitosan in 1% *d*-4 acetic acid in D_2O

The yields of the quaternized product obtained are reported based on the % weight fractions, which were calculated as the w/w percentage of the product (the quaternized derivative) and the reactant (chitosan derivative employed in the quaternization). Since there are greater numbers of primary amino groups available in unsubstituted chitosan

compared to any of its derivatives, the largest % weight fraction is theoretically possible for the unsubstituted chitosan (190.6%) compared to its derivatives. The quaternized derivatives were isolated using acetone precipitation of the product from a concentrated aqueous solution. This approach, in spite of being a time saver, has one inherent deficiency in the failure in recovering quantitative amounts of the product, some of which always remained dissolved in the precipitation medium (mother liquor). As a result, % weight fraction highly depended upon the solubility of the quaternized derivative in water (which was usually high) and on several experimental factors such as, volume of the aqueous solution of the derivative from which the product was precipitated, amount (volume) of acetone added during precipitation etc.

Table 9: Quaternization of various hydrophobic chitosan derivatives obtained by γ -lactone and anhydride addition

Entry	Chitosan derivative	% alkyl substitution	% weight fraction in Quat
1	<i>n</i> -heptanoyl-Chit	1.8	70.4
2	<i>n</i> -heptanoyl-Chit	2.4	109.5
3	<i>n</i> -heptanoyl-Chit	1.5	115.8
4	gluanh-Chit	10.1	115.4
5	gluanh-Chit	10.6	nd
6	γ -octanlact-Chit	low	85.3
7	γ -octanlact-Chit	low	139.7
8	γ -octanlact-Chit	low	52.8

nd: not determined

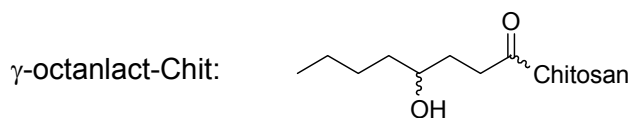
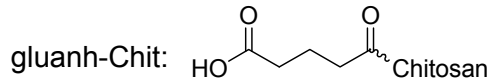
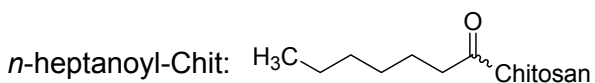


Table 10: Quaternization of various chitosan derivatives obtained by Bosch reduction methodology using aromatic aldehydes

Entry	Chitosan derivative	% aryl substitution	% weight fraction in Quat
1	Bz-Chit	17.5	98.6
2	Pip-Chit	12.4	126.2
3	Cinn-Chit	22.6	low
4	2-sulf-Bz-Chit	8.2	86.7
5	2-sulf-Bz-Chit	16.9	74.2
6	2-sulf-Bz-Chit	22.1	81.2
7	2-sulf-Bz-Chit	30.4	158.3
8	2,4-disulf-Bz-Chit	5.7	105.8
9	2,4-disulf-Bz-Chit	12.2	84.9
10	4-carbox-Bz-Chit	8.2	142.9
11	4-carbox-Bz-Chit	15.6	45.1
12	4-carbox-Bz-Chit	20.3	62.4
13	4-acetox-Bz-Chit	6.3	78.5
14	4-acetox-Bz-Chit	13.7	120.7
15	4-acetox-Bz-Chit	19.9	91.7

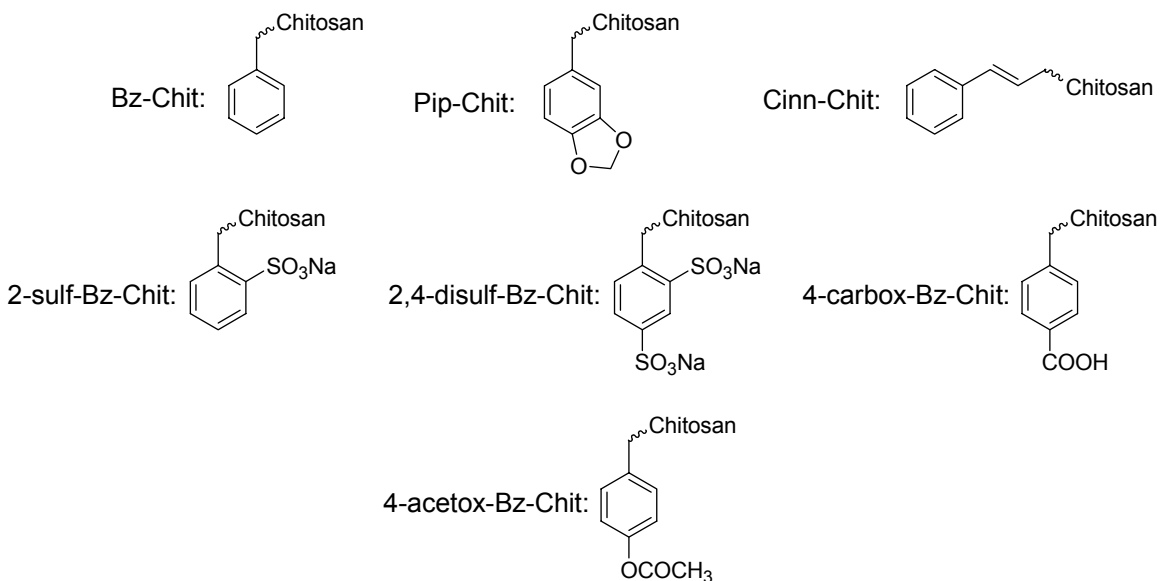


Table 11: Quaternization of various hydrophobic chitosan derivatives obtained by Bosch reduction methodology using aliphatic aldehydes

Entry	Chitosan derivative	% alkyl substitution	% weight fraction in Quat
1	<i>n</i> -octyl-Chit	5.5	96.0
2	<i>n</i> -octyl-Chit	5.8	nd
3	<i>n</i> -octyl-Chit	4.8	nd
4	<i>n</i> -octyl-Chit	6.8	137.8
5	<i>n</i> -octyl-Chit	11.1	96.4
6	<i>n</i> -octyl-Chit	35.7	147.7
7	<i>n</i> -octyl-Chit	58.5	nd

nd: not determined

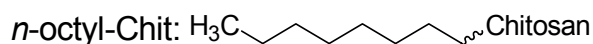


Table 12: Quaternization of various chitosan gels with aromatic cross-linker (CL)

Entry	Chitosan derivative [*]	%CL	% alkyl substitution	% weight fraction in Quat
1	CL(tereph)-Chit	4.8	none	103.0
2	CL(tereph)-Chit	49.0	none	109.1
3	CL(tereph)-Chit-gluanh	25.4	7.4	115.8
4	CL(tereph)-Chit-gluanh	18.5	6.8	115.4
5	CL(tereph)-Chit-gluanh	8.4	10.6	nd
6	CL(tereph)-Chit-glyox	23.5	(20)	113.6
7	CL(tereph)-Chit-glyox	12.5	(20)	83.5
8	CL(tereph)-Chit-glyox	7.3	(20)	53.3
9	CL(tereph)-Chit-glyox	5.9	(50)	nd

nd: not determined, * gel nomenclature used here provided in section 4.2.

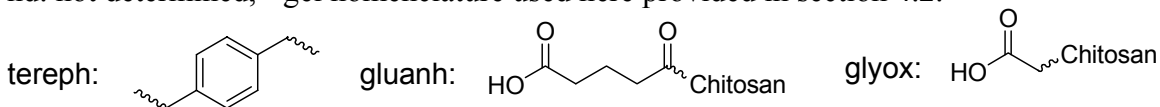
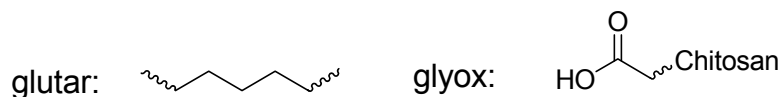


Table 13: Quaternization of various chitosan gels with aliphatic cross-linker (CL)

Entry	Chitosan derivative *	%CL	% alkyl substitution	% weight fraction in Quat
1	CL(glutar)-Chit	23.9	none	-
2	CL(glutar)-Chit-glyox	nd	(20)	98.0
3	CL(glutar)-Chit-glyox	nd	(50)	82.9

nd: not determined but anticipated to be close to 30.0 and 50.0 respectively for rows 2 and 3, * gel nomenclature used here provided in section 4.3.



CHAPTER 3. OPTIMIZATION OF REACTION CONDITIONS UNDER BOSCH REDUCTION METHODOLOGY

3.1 General Considerations

At the onset of the current project, one of the early objectives were to establish the optimal reaction conditions as applied towards the syntheses of targeted compounds under Bosch reduction methodology. There are several variables, for example, the reaction duration, temperature of the reaction and the added catalysts that could be tested for their effects on the progress of reaction. These optimization studies were carried out using two types of organic aldehydes, *n*-octyl aldehyde (Aliphatic, Table 14) and 3,4-dihydroxy benzaldehyde (Aromatic, Table 14). The choice of these aldehydes was made such that the former is insoluble under the aqueous reaction conditions employed, while the latter is soluble under same conditions. The optimization reactions were carried out with an assumption that any change in the outcome of the reaction between the representative compounds and chitosan as a result of modifications in reaction conditions would reflect in the same manner when other organic aldehydes were employed. It was found that the reactions of aliphatic aldehydes with chitosan under Bosch reduction methodology generally resulted in lower yields. The goal of this study was aimed at improving the yields under these circumstances.

3.2 Effect of β -Cyclodextrin as the Phase Transfer Catalyst

Cyclic oligomers of β -(1 \rightarrow 4)-D-glucopyranose are naturally occurring non-reducing carbohydrates that are produced from starch feed by certain types of bacteria in an enzymatic process (*cyclodextrin glycosyltransferase*).⁷³ Depending upon the number of

sugar units present per molecule, they are termed as α , β or γ cyclodextrins (6, 7 and 8 sugar units respectively). The molecule of β -cyclodextrin is a truncated donut (Figure 17) with a hollow cavity at the center.

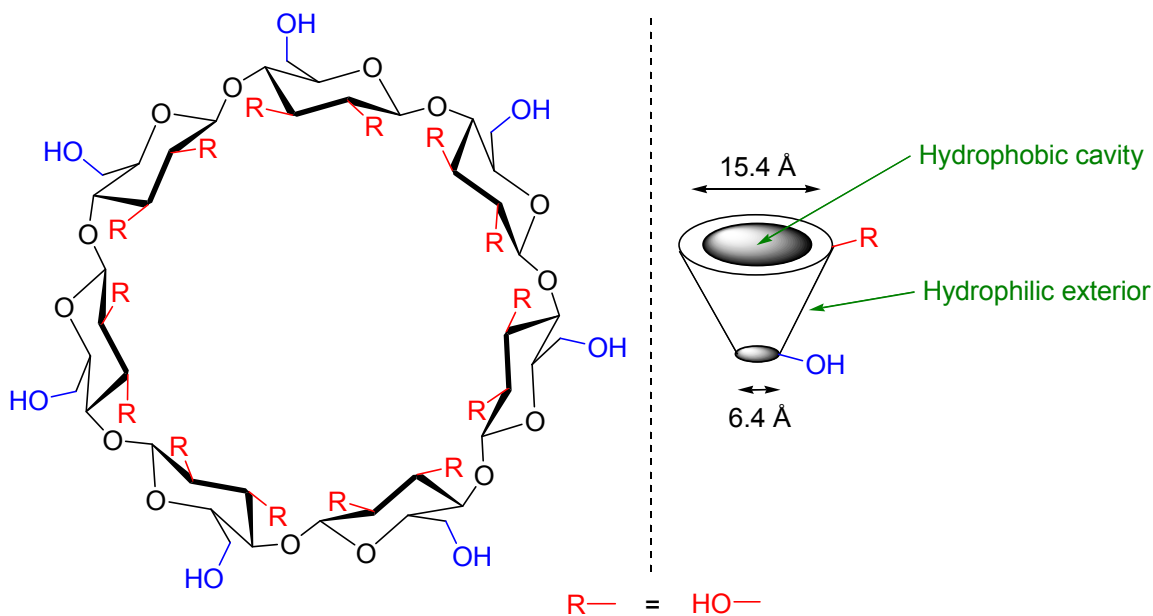


Figure 17: Chemical structure of β -cyclodextrin and shape schematic

β -cyclodextrin has been used extensively in the study of host-guest interactions which arise from the hydrophobic interior of the cavity.⁷⁴ The exterior surface of β -cyclodextrin is deemed hydrophilic due to the presence of hydroxy groups. The narrowest end of the center cavity in the molecule is about 6.4 Å in width and therefore may accommodate hydrophobic organic substrates along the core of the oligosaccharide. The non-covalent complexes resulting in this manner are termed as “inclusion complexes” where β -cyclodextrin acts as the host and the organic substrate nesting in the cavity as the guest. Several inclusion complexes of β -cyclodextrin with different hydrophobic organic substrates have been isolated and characterized.⁷⁵ The resulting supramolecular

assemblies often give rise to various molecular architectures such as rotaxanes, pseudo-rotaxanes etc.⁷⁶ The driving force for the formation of such complexes is ascribed to the hydrophobic-hydrophobic interactions. In addition to the small organic molecules, hydrophobic, linear side-chains of polymers have also been decorated with β -cyclodextrin. “Threading” of β -cyclodextrin in such cases was carried out with the ultimate purpose of forming main chain polyrotaxanes.⁷⁷

In the present project, β -cyclodextrin was employed as a possible phase transfer catalyst in the Bosch reduction methodology. It was anticipated that the mismatched polarities of chitosan dissolved in acidic solution (polar) and organic aldehydes (hydrophobic, non-polar) would result in phase separation of the two reactants under aqueous reaction conditions, which could adversely affect the outcome of the reaction. In the initial stages of the project, we therefore reasoned that due to the ambivalent nature of β -cyclodextrin, it may act as a catalyst by improving aqueous partitioning of hydrophobic aldehyde used in Bosch reduction methodology. With the help of control experiments carried out using aqueous solutions of β -cyclodextrin, it was established that the dialysis membrane used during the purification of products was porous to β -cyclodextrin. The product obtained in β -cyclodextrin catalyzed Bosch reduction was further quaternized to obtain water soluble hydrophobic chitosan derivatives. The results of these studies are given in Table 14 in the form of yield of polymer obtained.

All entries in Table 14 show a net weight gain in the resulting polymer compared to the initial weight of chitosan; indicating functionalization of chitosan. In most cases (Table 14; Entries 1, 3, 5 and 6) the data shows that reaction resulted in yields higher than theoretically possible.

Table 14: Reaction of chitosan with aldehyde in presence of 0.5 equiv. β -cyclodextrin at 60°C, followed by reduction with NaBH_3CN and quaternization

Entry	Molecular weight of chitosan used	Aldehyde used	Equivalents* of aldehyde	Polymer yield (g)	Theoretical yield ^a (g)
1	Low	Aliphatic	0.25	1.52	1.25 ^b
2	Medium	Aliphatic	0.25	1.27	1.88
3	High	Aliphatic	0.25	2.36	1.88
4	Low	Aromatic	0.25	1.76	1.90
5	Medium	Aromatic	0.25	3.27	1.90
6	High	Aromatic	0.25	2.38	1.90

^a considering no β -cyclodextrin binding to the chitosan derivative; ^b theoretical yield adjusted to reflect experimental error; * based upon 1g (5.2 meq.) chitosan

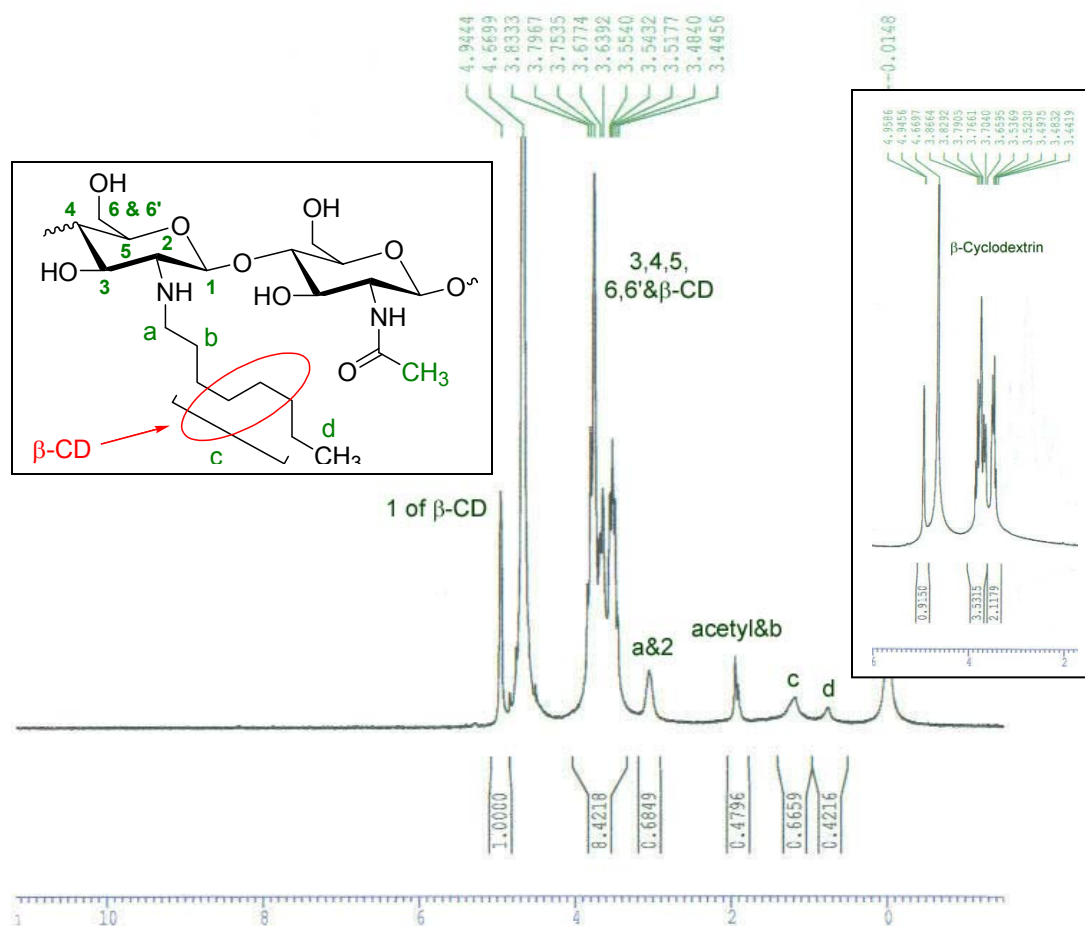


Figure 18: ^1H NMR spectrum of inclusion complex of *n*-octyl sidechain with β -cyclodextrin in 1% *d*-4 acetic acid in D_2O

This was attributed as due to the formation of inclusion complex of β -cyclodextrin with the hydrophobic sidechain in the product chitosan derivative. The molar composition of the complex (whether 1:1 or otherwise) could not be established based on its ^1H NMR spectrum (Figure 18). Further, this inclusion complex must have been stable enough to survive in its integrity during the steps followed in product isolation and purification (dialysis, acetone precipitation). The equivalents of β -cyclodextrin used in this method were 0.5 equiv. with respect to one sugar unit of chitosan. However, a molecule of β -cyclodextrin is composed of seven sugar units and consequently, an inclusion complex resulting from β -cyclodextrin and the hydrophobic alkyl sidechain of chitosan will result in higher signal intensity in ^1H NMR spectrum due to the ring hydrogens of β -cyclodextrin, compared to the ring hydrogens of chitosan. Due to this, as observed in Figure 18, the characteristic spectrum shape due to the ring hydrogens ($\delta \approx 3.4\text{--}3.8$) resembles closely to that observed for β -cyclodextrin (Figure 18; spectrum inset).

3.3 Time Required for the Completion of Reaction

Time dependent studies performed at two different temperatures are summarized in Table 15. The reaction studied for this purpose was the Schiff base formation reaction; the first process underway in Bosch reduction methodology (Scheme 15). Results obtained in Table 15, show temperature dependence of the reaction. Thus, reactions performed at room temperature indicate minimal time independence past the initial 72 hours. Association of β -cyclodextrin with the product is once again determined for reaction performed at room temperature (represented by yields greater than the theoretically possible value). The results obtained for reaction carried out at 60°C were

always lower than the theoretical yield. The reason for erratic yields at 60°C was not understood and a trend could not be realized with the help of the available data.

Nevertheless, it was clear from these studies that the Schiff base formation was complete within first 72 hours of reaction time. The reactions performed in the due course of the project strongly suggested that a time period of 6-10 hours was usually sufficient for the complete formation of Schiff base. As discussed under section 2.5.1 (*vide infra*), the formation of Schiff base could be visually observed and hence its formation and completion could be fairly assessed by visual inspection of the reaction.

Table 15: Reaction of chitosan with 2.0 equiv. *n*-octyl aldehyde, in presence of 0.5 equiv. β -cyclodextrin

Entry	Reaction time (hours)	Polymer yield (g) at RT	Polymer yield (g) at 60°C	Theoretical yield ^a (g)
1	72	2.16	0.63	1.02
2	96	2.23	0.72	1.02
3	120	2.09	0.24	1.02
4	150	1.92	0.46	1.02

^a based upon 0.6g (3.1 meq.) chitosan and considering no β -cyclodextrin binding to the chitosan derivative

3.4 Temperature Dependence of the Reaction

In order to optimize the reaction conditions for the reductive amination reaction, we decided to study the reaction feasibility at two temperature conditions. Temperatures in excess of 60°C were thought to be not suitable as higher temperatures may possibly increase the likelihood of aldehyde oxidation in addition to other side products of the reaction. On the other hand, temperatures below room temperature (RT) will probably have an adverse effect on the rate of reaction. Along with the effect of temperature on

the reaction outcome, we also verified the catalytic role of β -cyclodextrin in this reaction.

The results obtained are listed in Table 16.

Table 16: Reaction of chitosan with 2.0 equiv. *n*-octyl aldehyde followed by reduction with NaBH_3CN

Entry	Molecular weight of chitosan used	Reaction temperature °C	Equivalents of β -cyclodextrin*	Polymer yield (g)	Theoretical yield (g)
1	Low	RT	0.5	3.03	1.70 ^a
2	Low	60	0.5	0.84	1.70 ^a
3	Low	RT	0	1.00	1.70
4	Low	60	0	0.94	1.70

^a considering no β -cyclodextrin binding to the chitosan derivative; * based upon 1g (5.2 meq.) chitosan

A marked difference in temperature dependence is observed under the conditions with and without the use of β -cyclodextrin (Table 16; Entries 1 and 2). At higher temperature, yield of the product obtained under Bosch reduction methodology decreases more than 3 folds (Entry 2). During its mode of catalytic activity, β -cyclodextrin first forms a non-covalent inclusion complex with the hydrophobic aldehyde. This complex has a better solubility in aqueous medium compared to the aliphatic aldehyde itself. The interactions between β -cyclodextrin and aliphatic aldehyde in this complex are non-covalent, hydrophobic-hydrophobic type of interactions, and hence these interactions tend to be weaker and more susceptible towards further weakening at higher temperatures, a phenomenon very well observed and documented in literature.⁷⁵ As a result, at relatively high temperatures, the stability of this complex is greatly affected and under such circumstances β -cyclodextrin may turn ineffective, as a phase transfer catalyst. In the absence of added β -cyclodextrin as a catalyst, reactions at room temperature and at 60°C

show comparable results (Table 16; Entries 3 and 4). These results are obtained for the overall Bosch reduction methodology, which unlike Schiff base formation (discussed in Section 3.3, *vide infra*) shows no dependence on reaction temperature. This suggests that the overall Bosch reduction process does not gain by an increase in temperature. At higher temperatures, the yield of the substituted chitosan was lower compared to the reaction at room temperature (Table 16; Entries 4 and 5). This is probably due to the side reactions of the aldehyde at higher temperatures. An effect of β -cyclodextrin on the yield obtained in the Bosch reduction methodology can also be realized from Table 16 (compare Entries 1 and 3 and 2 and 4). In agreement with section 3.2 (*vide infra*), it can be seen that the yield of the product from the Bosch reduction methodology improved in presence of β -cyclodextrin in the reaction medium, but this effect is greatly pronounced only at room temperature. Based on Table 16, it can be concluded that β -cyclodextrin helps catalyze Bosch reduction methodology using chitosan, but it does so only at lower temperatures. It loses its catalytic activity at higher temperatures.

3.5 Effect of Organic Co-solvent on the Reaction

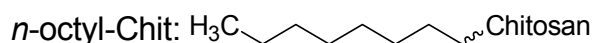
As discussed previously (section 2.5.1.1, *vide infra*), Bosch reduction methodology for aliphatic aldehydes was less efficient compared to when aromatic aldehydes were used (section 2.5.1.2, *vide infra*), as evident from the difference in % substitution obtained. Based on these findings, we reasoned out the difference in the results as a direct consequence of the reactivity trend of the two types of aldehydes (*viz.* aliphatic and aromatic) under heterogeneous reaction conditions. In order to improve the reactivity and hence % substitution using aliphatic aldehydes, we employed DMF (dimethyl formamide) as the co-solvent during the process. Doing so was thought to “keep” the

aldehyde dissolved in the reaction medium, thereby making the process homogeneous and hence provide for an opportunity for better interaction between the two reactants. Synthetic procedures involving use of DMF are usually hampered by the fact that its separation from the reaction product is often tedious, making the purification process lengthy and difficult. However, we thought this issue to be not of major concern in our case, since during the process of dialysis used in the purification of our macromolecular product, most of the solvent DMF would separate out from the reaction mixture by diffusion. Finally, during acetone precipitation used in the isolation of the product, any remainder traces of DMF would wash out, leaving behind the product free of any residual DMF. It was therefore thought, it would be reasonable to use a co-solvent in an attempt to enhance the degree of substitution and also gain better control over the extent of substitution.

Results obtained in using an organic co-solvent under Bosch reduction methodology are tabulated in Table 17. The aliphatic aldehyde (*n*-octyl aldehyde) was allowed to react with chitosan under similar protocol as represented in section 2.4.2.1 (*vide infra*), but in the presence of DMF (150mL), added prior to the aldehyde.

Table 17: Bosch reduction in presence of DMF as co-solvent

Entry	Derivative	% substitution targeted	% substitution calculated	% recovery
1	<i>n</i> -octyl-Chit	10.0	6.8	75.4
2	<i>n</i> -octyl-Chit	20.0	11.1	86.8
3	<i>n</i> -octyl-Chit	50.0	35.7	69.1
4	<i>n</i> -octyl-Chit	100.0	58.5	78.4



The reaction in presence of DMF resulted in the hydrophobic chitosan derivative free of any residual DMF as verified by the ^1H NMR spectrum. The % recovery for these derivatives was moderate (Table 17; Entries 1, 3 and 4) to high (Table 17; Entry 2), however employing a co-solvent resulted in very high levels of substitution with the hydrophobic moiety (Table 17; Entries 3 and 4). Figure 19 provides the correlation between % substitution targeted and % substitution obtained using this approach.

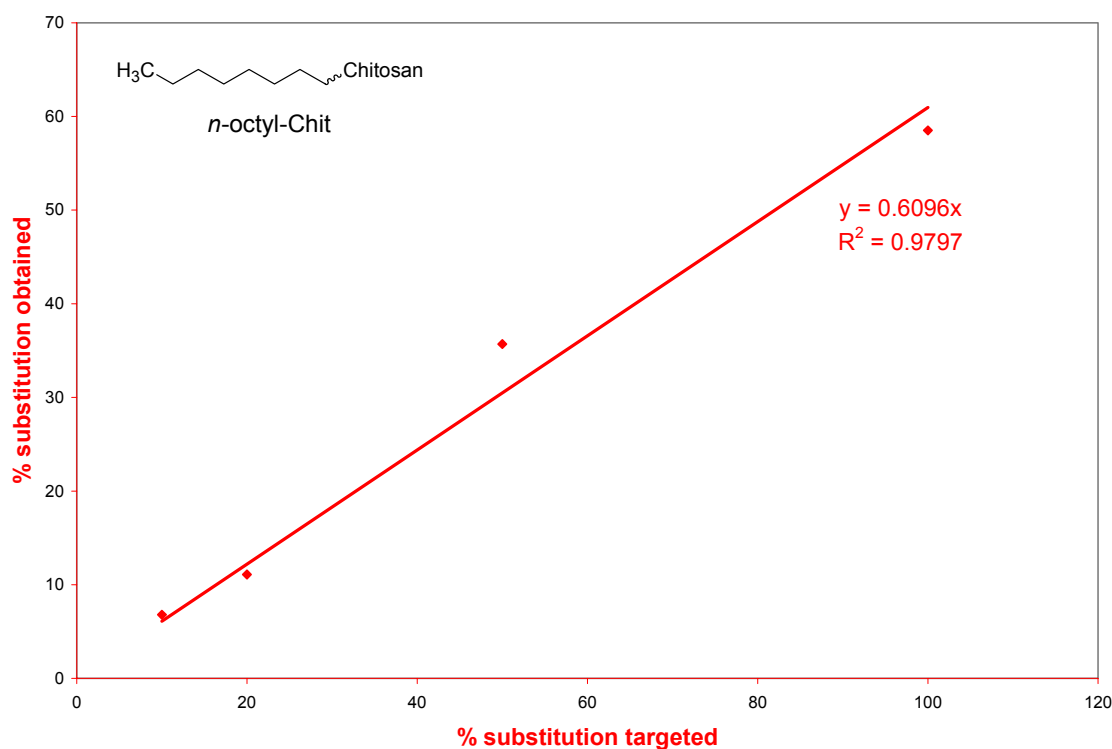


Figure 19: Substitution control using an organic co-solvent (DMF)

A linear correlation with a high R-squared value obtained in Figure 19 strongly indicates excellent control of substitution in Bosch reduction methodology using long chain aliphatic water-immiscible aldehydes. These findings also suggest that, organic co-solvent (DMF) may aid in the substitution control in reactions of chitosan with hydrophobic γ -lactones and anhydrides (section 2.5.2, *vide infra*).

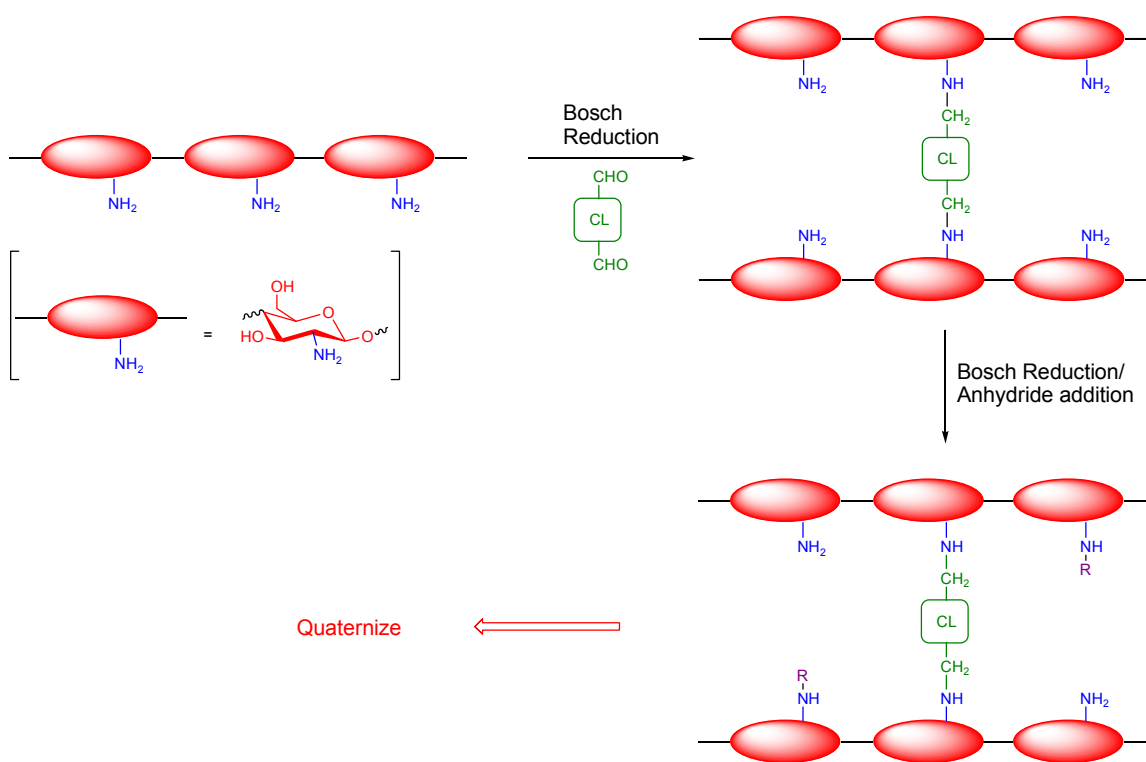
CHAPTER 4. CROSS-LINKED CHITOSAN DERIVATIVES

4.1 Introduction

Chitosan gels obtained by acylation of the free amine groups have been documented in literature.⁷⁸ These studies have shown that under dynamic measurement conditions, the time of gelation decreases with increasing concentration of the chitosan in the reaction mixture starting with a polymer concentration of 1% (w/w). Further, the time to the onset of gelation was found to be proportional to the chain length of the acyl group incorporated on the chitosan backbone. The gelation of acyl-chitosan derivatives is described as due to reduced solubility of the polymer chain resulting from *N*-acylation. A gelation mechanism for *N*-acylated chitosan derivatives has been proposed in a separate communication by the same authors.⁷⁹ In another report, polyamphoteric hydrogels were prepared by simultaneous graft co-polymerization of 2-acrylamido-2-methyl-propane sulfonic acid and *N*-isopropyl acryl amide onto chitosan at 30°C using potassium persulfate as the initiator and *N,N,N',N'*-tetramethylethylene diamine as the accelerator.⁸⁰ Polyelectrolyte complexation between chitosan and the sulfonic acid groups present in the graft co-polymer was used to alter hydrophobic/hydrophilic balance with in the polymer thereby modifying properties of the resulting hydrogel. The resulting hydrogels showed higher values of lower critical solution temperature, a transition representing change of state from swollen to dehydrated, collapsed state. Thermoreversible polymers (and hydrogels) are pursued for potential applications in biomedical field such as implant materials,⁸¹ drug delivery systems⁸² and tissue engineering scaffolds.⁸³ In addition to the covalent systems mentioned above, hydrogels prepared from polyelectrolyte complexes

of chitosan and hyaluronic acid have been shown to exhibit good swelling behavior which was observed to increase with increasing content of chitosan in the polyelectrolyte complex.⁶⁸ Such materials showed a non-fickian behavior and demonstrated both, diffusion and relaxation controlled water sorption.

Chitosan gels were synthesized in the present work by chemical cross-linking of sugar residues (Scheme 19). The cross-linking was achieved under Bosch reduction methodology using terephthalaldicarboxaldehyde or glutaraldehyde as the hydrophobic cross-linkers (CL). In most cases, following the cross-linking, polymers obtained were further substituted using either, glyoxylic acid under Bosch reduction methodology or with glutaric anhydride. The cross-linked polymer derivatives thus obtained were further quaternized as necessary using Quat-188 (Scheme 18).



Scheme 19: Synthesis of cross-linked chitosan derivatives

The cross-linking achieved in this manner could be either intra-molecular or intermolecular in nature. However, an increase in the concentration of dialdehyde (CL) decreased onset of the gelation concentration; an observation highly suggestive of higher cross-linking. The quaternized gels obtained were tested for their antibacterial activity and the results obtained are discussed in Chapter 5.

4.2 Chitosan Cross-linking Using Terephthaldicarboxaldehyde

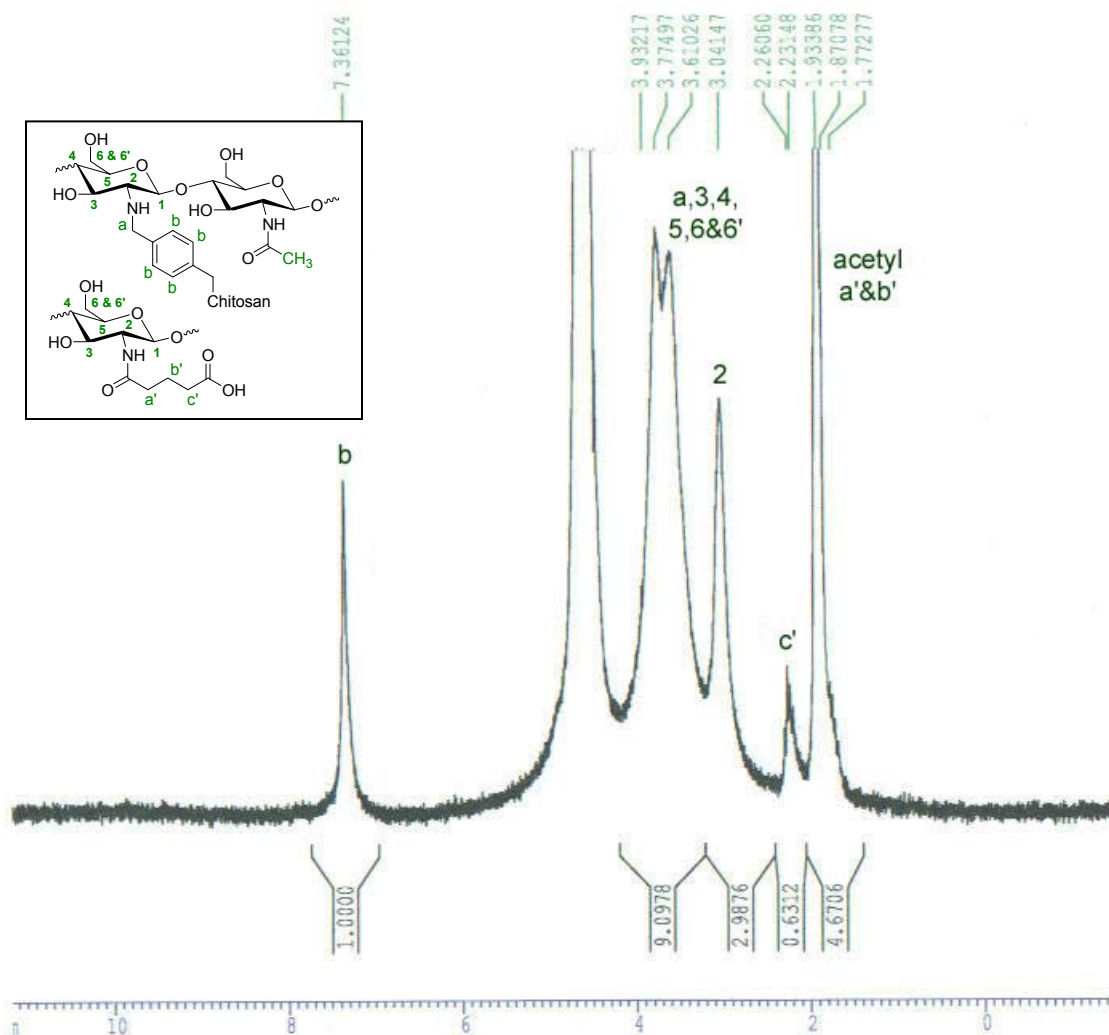
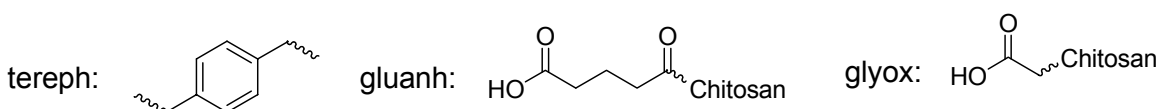


Figure 20: ^1H NMR of a representative chitosan gel in 1% d -4 acetic acid in D_2O

Hydrophobic aromatic cross-linking using terephthalaldehyde proceeded well with good control of substitution. Cross-linked chitosan obtained using aromatic dialdehyde, when dissolved in 1% acetic acid resulted in gels, viscosity of which was dependent upon the extent of cross-linking. Unlike *N*-acyl chitosan gels, which require degree of substitution for the acyl groups of 0.66-0.75 (with respect to the amino groups in chitosan) for any gel formation to occur,⁷⁹ terephthalaldehyde mediated cross-linking was observed to result into gels at as low a degree of substitutions as 0.05. A typical ¹H NMR spectrum of a cross-linked chitosan polymer formed by cross-linking with terephthalaldehyde followed by substitution with glutaric anhydride is given in Figure 20. Table 18 provides different chitosan gels prepared and % cross-linking and substitution obtained in each case.

Table 18: Chitosan gels synthesized

Entry	Gel	% CL		% subst. (targeted)	% recovery
		obtained	targeted		
1	CL(tereph)-Chit	4.8	5.0	none	78.3
2	CL(tereph)-Chit	49.0	50.0	none	82.9
3	CL(tereph)-Chit-gluanh	25.4	30.0	7.4(20)	66.4
4	CL(tereph)-Chit-gluanh	18.5	20.0	6.8(20)	81.9
5	CL(tereph)-Chit-gluanh	8.4	10.0	10.6(20)	94.9
6	CL(tereph)-Chit-glyox	23.5	30.0	(20)	72.7
7	CL(tereph)-Chit-glyox	12.5	20.0	(20)	84.5
8	CL(tereph)-Chit-glyox	7.3	10.0	(20)	92.7
9	CL(tereph)-Chit-glyox	5.9	10.0	(50)	59.4



Nomenclature assigned to different gels in Table 18 is as follows- CL(cross-linker)-Chit-(substituent). For example, a gel prepared by using terephthalaldehyde as the cross linker followed by reaction with glutaric anhydride is represented here as CL(tereph)-Chit-gluanh, etc. The extent of cross-linking (%CL) and substitution as reported in the table above was determined based on ^1H NMR, using Equation 4 with appropriate modifications. Gels resulting from terephthalaldehyde (Table 18; Entries 1 and 2) showed excellent cross-linking control (represented by %CL values). In case of the substitutions on the gel using glutaric anhydride (Table 18; Entries 3,4 and 5), signal due to hydrogens α to the resulting carboxylic acid ($\delta = 2.26$, Figure 20) formed by ring opening of anhydride forming ω -carboxylic acid moiety was used to evaluate % substitution. These entries clearly indicate a sharp contrast between the substitution tendencies of glutaric anhydride compared to *n*-heptanoic anhydride as discussed in section 2.5.2 (*vide infra*). The possible explanation for greater reactivity of glutaric anhydride under the same protocol is the ring strain release brought about upon substitution. However, % substitution obtained under same levels of targeted substitution using glutaric anhydride was found to vary among different gels at different levels of aromatic cross-linking. Further, no trend could be observed in the levels of substitution obtained due to glutaric anhydride in such cases. Aromatic substitution in these gels was found to show a logarithmic relationship between % substitutions targeted and obtained (Figure 21).

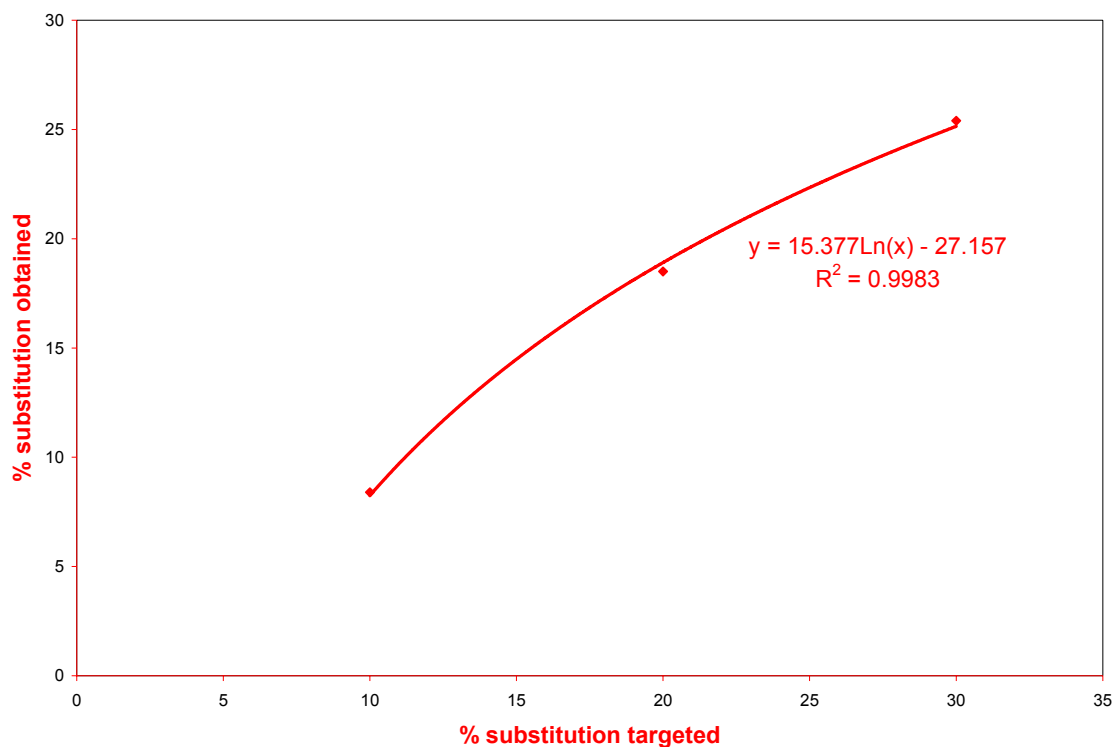


Figure 21: Substitution control in chitosan-terephthaldicarboxaldehyde gels of type CL(tereph)-Chit-gluanh

Substitution on gel using hydrophilic substituent was performed using water soluble glyoxylic acid (Table 18; Entries 6, 7, 8 and 9). The extent of substitution of carboxymethyl moieties in the resulting gel could not be determined using ^1H NMR method, since the proton resonance due to such substituent overlaps with those due to the sugar protons from the chitosan backbone. However, as will be discussed in section 4.3 (*vide infra*), water soluble aliphatic aldehydes resulted in % substitutions that were close to the targeted values. As a result, it is very likely that % substitutions obtained with glyoxylic acid under Bosch reduction methodology were close to the targeted values. When cross-linking and substitution was carried out in one step using excess of glyoxylic acid compared to terephthaldicarboxaldehyde (Table 18; Entry 9), the resulting gel

showed reduced presence of the cross-linker. In this case, with an excess of water soluble aldehyde, it is likely that the Bosch reduction initially occurred mainly with glyoxylic acid. Under this pathway, it is possible that the resultant substituted derivative, carboxymethyl chitosan, with high levels of acidic substituents may have altered the reactivity of the remainder free amino groups within the polymer backbone, resulting in reduced cross-linking.

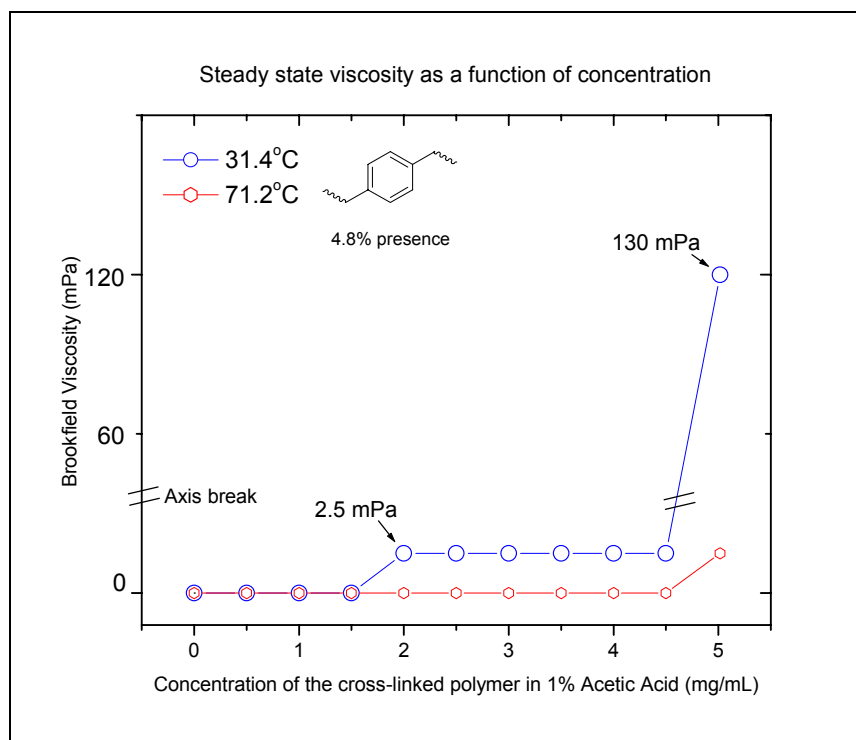


Figure 22: Viscosity dependence of chitosan-terephthaldicarboxaldehyde gel (4.8% presence) as a function of concentration at two temperatures

Figure 22 and Figure 23 represent viscosity dependence as a function of concentration for the gels of type CL(tereph)-Chit, prepared in 1% acetic acid. The viscosity measurements were performed on a Brookfield viscometer using S-27 spindle at rotation speed of 100 rpm. The two gels with different extent of cross-linking (presence of the aromatic moiety- 4.8% and 49% for Figure 22 and Figure 23 respectively measured using

^1H NMR) exhibited different onset of gelation concentrations. Thus, highly cross-linked sample (presence of aromatic moiety- 49%) demonstrated an onset of gelation at around 3mg/mL compared to 4.5mg/mL for the gel with lower degree of cross-linking (4.8%) at 31.4°C. Gel with 4.8% aromatic substitution showed no apparent gelation up until 5mg/mL at higher temperature (71.2°C). In addition, temperature dependence of the gel viscosity can be seen from these figures. An increase in temperature resulted in reduced viscosity of both the gels.

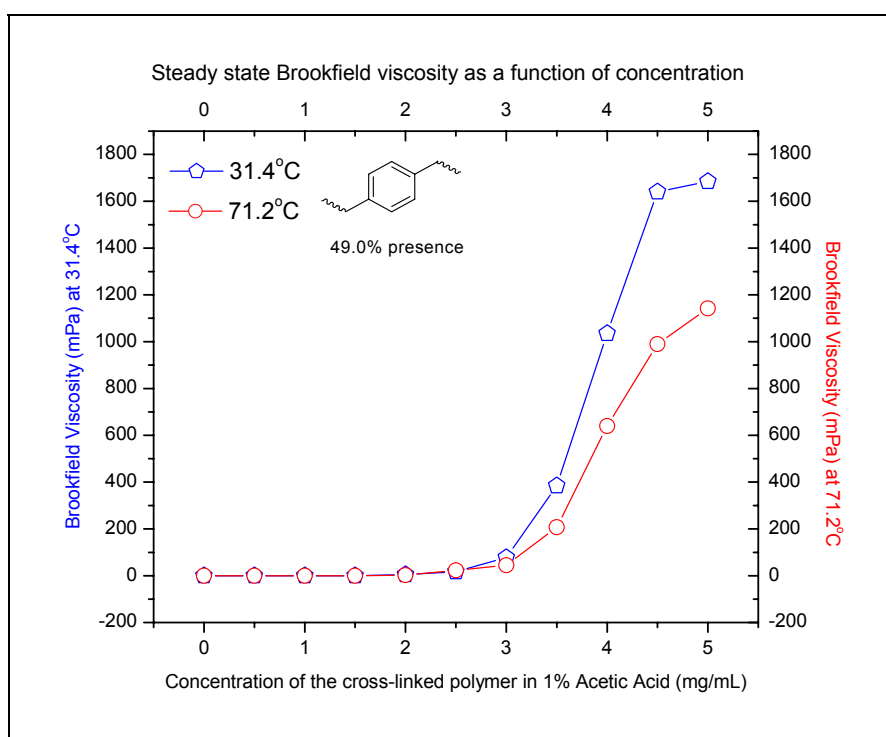


Figure 23: Viscosity dependence of chitosan-terephthaldicarboxaldehyde gel (49.0% presence) as a function of concentration at two temperatures

At any given concentration and temperature past the onset of gelation, the absolute viscosities of the two gels were quite different. Solutions of gel with greater cross-linking were always more viscous compared to the solutions of lower cross-linked material at the same concentration and temperature. However, on absolute terms, change

in the gel viscosities near onset of gelation due to a temperature increase from 31.4°C to 71.2°C as a function of gel concentration are comparable for the differently cross-linked gels as apparent from the initial slopes of two curves in Figure 24.

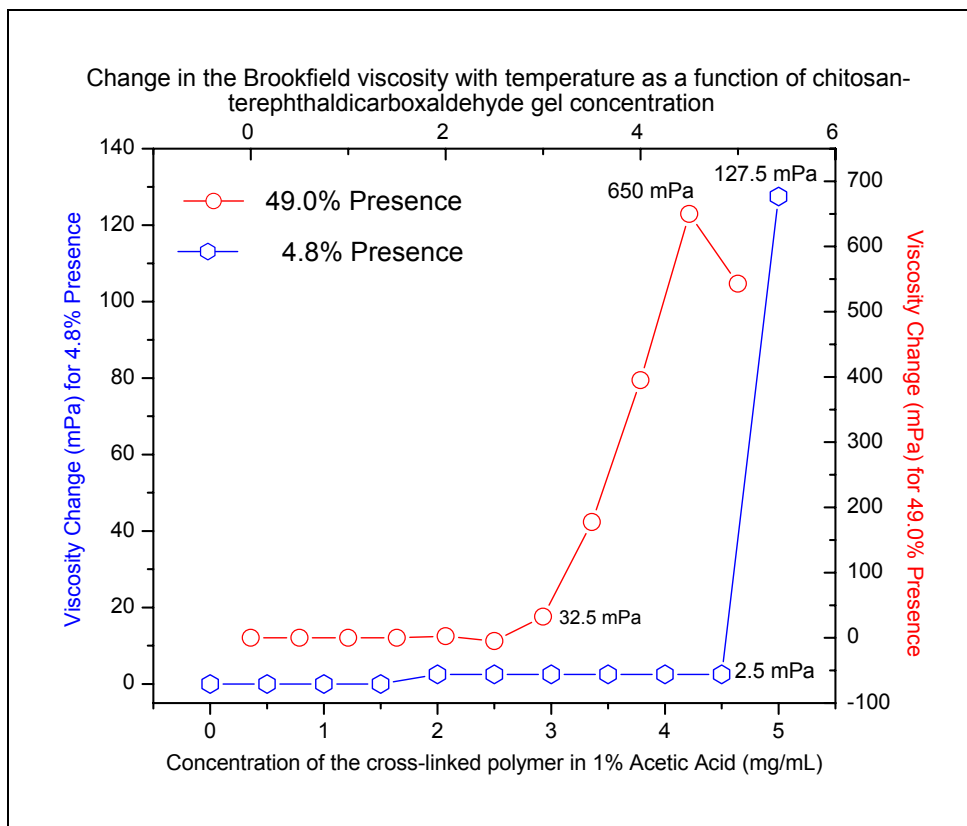


Figure 24: Change in the viscosity of chitosan-terephthaldicarboxaldehyde gel associated during temperature increase from 31.4°C to 71.2°C as a function of concentration

Based on these observations, it appears that the inter-chain aggregation resulting into the formation of a gel in these systems is caused by hydrogen bonding interactions between the polymer chains. It can also be seen from Figure 24 that the gel containing 49% aromatic substitution showed a smaller viscosity change with the temperature increase past the concentrations required for complete gelation (4.5mg/mL, Figure 24); the trend observed in the change in viscosity receded past complete gelation. This is

indicative of a greater temperature tolerance and rigidity of the network past the gelation point.

During the syntheses of various chitosan based gels, one such sample showed excellent water-sorption capacity of greater than 2500% of the weight of dry material. The gel was prepared by first substituting chitosan with glutaric anhydride under anhydride addition conditions (20.0% targeted substitution) followed by the cross-linking of the resulting derivative with 20mol% terephthalaldehyde under Bosch reduction protocol. The resulting cross-linked material exhibited spontaneous aggregation resulting into a thick gel which could not be analyzed using ^1H NMR spectroscopy due to difficulties associated with liquid sample preparation of the gel.

4.3 Chitosan Cross-linking Using Glutaraldehyde

Glutaraldehyde was employed as a flexible hydrophobic cross-linker in preparation of chitosan gels. A representative ^1H NMR of the resulting gel is given below (Figure 25). The appearance of a broad signal assigned to the aliphatic hydrocarbon chain of glutaraldehyde ($\delta = 1.45\text{-}1.81$) is likely due to the aggregation and appeared in all of the gels prepared by glutaraldehyde cross-linking. The % cross-linking based on the ^1H NMR spectrum was found to be 23.9% (targeted 25.0%). The degree of cross-linking was determined by first subtracting the contribution due to the residual acetyl groups in chitosan from the signal integral due to “b and acetyl” ($\delta \approx 1.45\text{-}1.81$) followed by comparison of the resultant signal intensity with the sugar signal ($\delta \approx 3.50\text{-}3.95$) due to five protons, in an approach similar to Equation 4. High levels of cross-linking (cross-linking efficiency) observed in the gels prepared using glutaraldehyde are likely due the homogeneous reaction conditions during their preparation. Glutaraldehyde derived

chitosan gels were further substituted by glyoxylic acid under Bosch reduction methodology and the results obtained are given in Table 19.

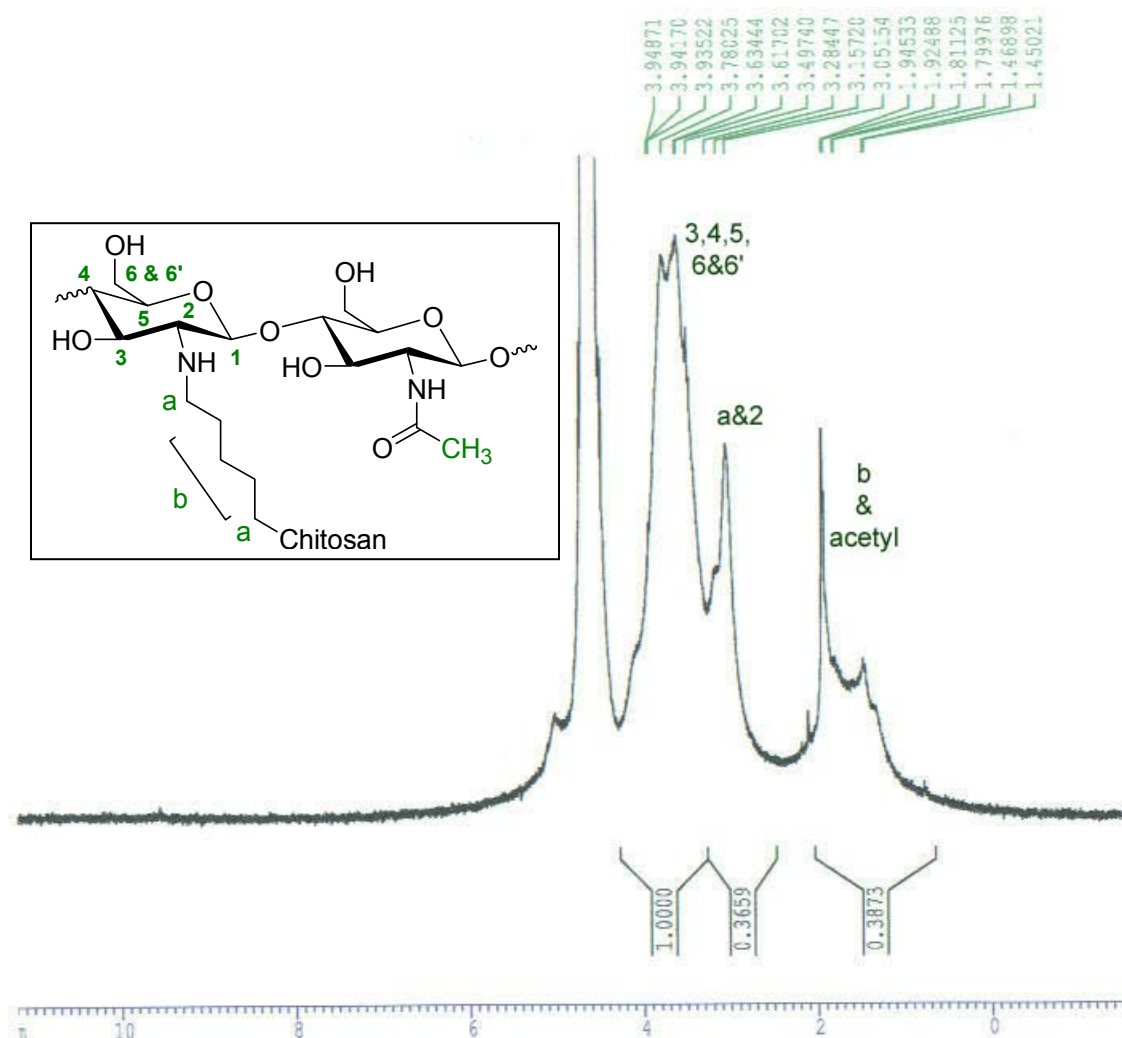


Figure 25: ¹H NMR of chitosan glutaraldehyde gel in 1% *d*-4 acetic acid in D₂O

Chitosan gels prepared using glutaraldehyde showed good % recovery. In entries where carboxymethyl substitution of glutaraldehyde cross-linked gel was carried out; the extent of substitution (% substitution) due to carboxymethyl moieties and the extent of cross-linking (% CL) could not be established using ¹H NMR spectroscopy, since the proton resonance due to these substituents overlaps with those due to the sugar protons

from the chitosan backbone. This further invalidates the calculations used in determining the extent of cross-linking. Given the high cross-linking with water-soluble glutaraldehyde (Table 19; Entry 1), it is likely that % substitutions obtained with glyoxylic acid were close to the targeted values. A high % carboxymethylation may have resulted in mono as well as di-substitutions.²⁷

Table 19: Chitosan gels prepared using glutaraldehyde as the cross-linking agent

Entry	Gel	% CL		% subst. (targeted)	% recovery
		obtained	targeted		
1	CL(glutar)-Chit	23.9	25.0	none	88.4
2	CL(glutar)-Chit-glyox	nd ¹	30.0	(20)	90.8
3	CL(glutar)-Chit-glyox	nd ¹	50.0	(50)	98.7

¹ not determined; but anticipated to be close to the targeted value



CHAPTER 5. ANTIBACTERIAL ACTIVITY OF HYDROPHOBIC CHITOSAN DERIVATIVES BEARING QUATERNARY AMMONIUM FUNCTIONALITY

As discussed briefly in section 1.4.1, chitosan and its derivatives exhibit good to excellent antibacterial and antifungal properties. This in conjunction with other characteristics of chitosan such as low toxicity toward mammalian cells,⁸⁴ biocompatibility, biodegradability and natural renewable occurrence has attracted interest in recent years in research and development aimed towards widening the scope of chitosan derived antibacterials. However, one major obstacle in the application of chitosan and some of its derivatives is the insolubility of chitosan in aqueous solutions at pH 7. This is undesirable since many of the applications mainly of biological relevance may not be realized if the chitosan solutions are not tolerant of the physiological pH. In addition to the solubility issues, chitosan solutions are often viscous and it tends to coagulate with proteins under physiological pH conditions.⁸⁵ Several reports in literature have addressed the issue of water insolubility by preparing functional derivatives of chitosan by chemical modifications.

5.1 Background

The antimicrobial activity of chitosan has been observed against a wide variety of microorganisms including fungi, algae and bacteria. However, the antimicrobial action is greatly influenced by many factors such as the source of chitosan, degrees of deacetylation and polymerization, the microorganism, pH of the growth medium and environmental conditions (such as moisture content, natural nutrient availability to the microorganism etc.).⁸⁵ The antimicrobial activity of native chitosan is higher at around pH 6 (compared to at pH 7.5), when most amino groups remain protonated (pK_a value of

chitosan= 6.5).⁸⁶ Chitosan has been utilized in soil amendment, seed treatment and the foliar treatment to control fungi growth.⁸⁷ In addition, chitosan has been successfully used as food wraps.⁶ Chitosan films are tough, elastic and durable; comparable to many of the medium-strength commercial polymers.⁸⁸ When applied as a coating over fruits and produce, the film controls the influx of moisture and oxygen and also reduces transpiration loss and delays ripening process while preserving the food stock. Biodegradation of chitosan produces known chemical intermediates that are known to be harmless to humans and the environment. Chitosan and its derivatives have several advantages over other types of disinfectants because of higher antibacterial activity, broader spectrum of activity, a higher killing rate and lower toxicity. However, given the differences in activities due to various chitosan derivatives for different microorganisms, it is rather difficult to predict the specific chemical modifications in chitosan necessary to deliver the desired antimicrobial activity. Additionally, the activity of any given chitosan derivative is dependent upon the host microorganism. Nevertheless, the antimicrobial property of chitosan has facilitated its application in a variety of fields including food science, agriculture, medicine, pharmaceuticals and textiles.⁸⁹

5.1.1 Chitosan and Its Derivatives as Antimicrobials

Several derivatives of chitosan reported in the literature show greater solubility under neutral conditions and an enhanced activity compared to native chitosan. The simplest of these derivatives are the salts with acids. In this regard, chitosan-pyrithione [(chitosan salt with omadine acid (2-mercapto-pyridine-*N*-oxide)] showed a broad spectrum antibacterial and antifungal activity that was far better than simple salts like chitosan-acetate.⁹⁰ However, such salts even though soluble under neutral pH conditions, they

often tend to form a precipitate of chitosan under basic conditions and hence have limited applicability in products of commercial interest. Quaternary ammonium derivatives of chitosan show enhanced activity compared to the native chitosan.⁹¹ Quaternization also generates a permanent positive charge on the polymer backbone which renders water solubility to such derivatives under neutral pH conditions. The antibacterial activity due to these salts was found to be dependent upon the counter anion.⁹² Thus, an iodide salt was found more active compared to the chloride and hydroxide. Amongst different quaternary ammonium derivatives of chitosan, the one obtained by the treatment with glycidyl trimethylammonium chloride showed very high antibacterial activity.

Carboxymethylation of chitosan provides water soluble derivatives. However, only *O*-carboxymethyl chitosan was found to exhibit antibacterial activity better than the native chitosan but not the *N,O*-carboxymethylated derivative.³⁸ This was explained as due to reduced availability of -NH_2 groups in the latter derivative. *N*-sulfonated chitosan derivatives form another class of water soluble, chitosan based antibacterials.²⁸ However, the optimal sulfonyl substitution in such derivatives in order to obtain best possible activity was different for different bacteria. In addition, sulfonyl substitution past the optimal level resulted in reduced activity. This observation was attributed to reduced interactions with the cell by a greater negative character of the sulfonated derivative past the optimal levels of substitution.

5.1.2 Mode of Action

Native chitosan exhibits much more pronounced activity compared to chitin.⁴² This is usually described as due to the greater availability of primary amino groups in the former. Under mildly acidic conditions (\approx pH 6), the amino groups acquire a positive charge,

which is usually associated with the demonstrated activity. The exact mode of interaction between chitosan, its derivatives and the microorganism is still unknown, but different mechanisms have been proposed in the explanation of antimicrobial activity. It is believed that the polycationic nature of chitosan initiates binding with the cell membrane by means of electrostatic attraction with negatively charged microbial cell membrane. It was observed that at lower concentrations (0.2mg/mL), chitosan caused agglutination of bacterial cell. Distortions and fraying of the bacterial outer cell wall upon treatment with chitosan has been observed with the help of TEM (Transmission Electron Microscopy) analyses in the case of *E. coli*.⁹³ TEM analyses also revealed that the cytoplasmic membrane of bacteria was detached from the inner part of the cell wall after chitosan treatment. Once bound to the cell surface, chitosan is thought to affect membrane permeability which results into the leakage of proteinaceous material and other intracellular constituents of the microbial cell causing death due to the loss of essential fluids.⁹⁴ Chitosan is also thought to act as a chelating agent that binds trace metal ions present in the intracellular fluid, which play essential role in the biochemistry of microbes.⁹⁵ Deficiency of such metal ions may inhibit production of toxins, enzymes and the microbial growth. Chitosan is also observed to bind DNA and inhibit mRNA and protein synthesis upon penetration into the nuclei of fungi.⁹⁶

Modes of activity proposing interactions with the intracellular components (and nuclear components) require that chitosan be hydrolyzed in order to produce fragments low in MW for effective penetration and transport across the cell membrane of the microorganism. However, chitosan interaction with the nuclear components during the course of its activity is still debated. Studies have shown that very low MW (≈ 2000)

chitosan actually accelerates the bacterial growth.⁹⁷ It was proposed based on this finding that antimicrobial action due to chitosan is mainly governed by the suppression of the metabolic activity of the bacteria by blocking nutrient permeation through the cell wall rather than the inhibition of nucleic constituents. Often the antimicrobial effect due to chitosan is realized due to a combination of various triggers. For example, inhibition of the fungal growth in tomato and strawberries in the presence of chitosan is attributed as due to the reduction of aflatoxin, elicitation of phytoalexin and phenolic precursors, enhanced production of chitinases and other factors crucial in plant defenses.⁹⁸ At present, the signaling pathway from the point of chitosan-microorganism interaction to that at which the final response is drawn from the host cells is not well understood. However, it is known that chitineous substances and other oligosaccharides are natural defense systems in many plant species.⁹⁹ It is important to note that the activity of chitosan is highly dependent upon the pH of the local environment. For example, the antibacterial effect of chitosan at pH 4.8 was found non-existent at pH 7.6.⁹⁴

5.1.3 Factors Affecting Antimicrobial Activity

Several intrinsic (associated with the constitution of the macromolecule) and external parameters govern and control the antimicrobial activity due to chitosan and its derivatives. The unit monomer of chitosan, 2-amino-2-deoxy-D-glucopyranose as a hydrochloride salt does not exhibit any inhibition against several bacteria, including *E. coli* and *S. aureus*.⁴¹ This suggests that antibacterial action of chitosan could be a co-operative effect of several units acting against the bacteria. A review of available literature does not lead to any conclusive pattern establishing the relationship between the molecular weight of chitosan used and its activity. However, it seems clear that high

MW chitosan (>500000) shows reduced activity compared to lower MW material which is usually attributed to the difficulties in diffusion for highly viscous solutions of high MW chitosan during the tests.¹⁰⁰ Further, the activity seems to depend on the microorganism assessed. For example, medium and high MW chitosan exhibited higher activities against *Bacillus circulans* compared to chito-oligosaccharides (DP= 2-30).²⁹ However the activity reversed when tests were performed on *E. coli*. When comparing the trend against *E. coli*, the activity seems to increase with the MW, until it reaches a certain value (MW= 30000), after which the activity starts declining.⁹³

Unlike the lack of any observable trend between the MW of chitosan and its activity against the microorganisms, the degree of deacetylation (DD) in chitosan shows a direct relationship with the activity exhibited.³⁸ This is due to a greater availability of potentially cationic sites along the polymer backbone with the increase in DD. In a related situation, chitosan antimicrobial activity was found greater at lower pH values than those at higher.⁹⁴ Effect of temperature on the antibacterial activity indicated that chitosan shows greater inhibition at 37°C than at 0°C.⁹⁴ Polyanionic substances (for example sodium polygalacturonate) formed tight complexes with chitosan under mildly acidic conditions. This resulted in the reduced activity of chitosan against *Glycine max*.¹⁰¹ Presence of certain free metal ions (particularly alkaline earth metals) adversely affected the chitosan activity, possibly by reducing the availability of free amino groups through complexation.⁹⁴

5.2 Experimental

Antibacterial activity of various chitosan derivatives was established using Minimum Inhibitory Concentration (MIC) method. The assessments were performed by test well method using liquid culture of bacteria.

5.2.1 Materials and Miscellanies

Microorganisms used in the present study were *E. coli* (ATCC 25922) and *S. aureus* (ATCC 29213). The stock solutions of microorganisms were stored at -80°C. Phosphate buffer used in the assessments was prepared from potassium phosphate monobasic (ACS crystal) and potassium phosphate dibasic (ACS powder), both purchased from Fluka in the highest level of purity. Antibacterial assessments were performed in sterile, translucent Costar[®], 96 well cell culture cluster plates manufactured by Corning Inc. Polystyrene sterile Falcon[®] tubes (6mL and 14mL capacity) manufactured by Becton Dickinson and Co. were used to prepare bacterial solutions as well as solutions of antimicrobial agents at different concentrations. Difco[™] Nutrient Broth (referred in the protocol as NB) manufactured by Becton, Dickinson and Company, MD was used for bacterial inoculations. Spectrophotometer used in establishing the bacterial cell density was Ultrospec 4050 manufactured by LKB Biochrom, and the optical density (OD) measurements were performed in disposable polystyrene cuvettes. Assessments were performed starting with sterile solutions and working in a sterile environment. In order to help maintain the sterility during the assessments, a clean bench-space was sprayed with sufficient ethanol to cover the surface and wiped dry. In addition, assessments were carried out in the proximity of heat convection generated with the help of a Bunsen burner. All the solutions and accessories (micropipette tips, troughs etc.) used during the

assessments, except those containing microorganisms and the antibacterial agent, were autoclaved at 121°C for 20 minutes prior to their use. Solutions of antibacterial agents and microorganisms were prepared in previously autoclaved, sterile aqueous phosphate buffer. When conducting the antibacterial assessments, it is recommended to use freshly prepared bacterial stocks and so was done in all the assessments here.

5.2.2 Test Protocol

Each antibacterial agent was dissolved in sterile phosphate buffer to make *ca.* 8 stock test solutions with concentrations ranging from 4µg/mL to 512µg/mL in the multiples of 2 starting from the first. NB solution was made at a concentration of 8% (w/v) in phosphate buffer. Prior experiments conducted by Dr. Martha Juban (of LSU protein facility, College of Basic Sciences, LSU) determined the level of OD required from the bacterial solutions in order to attain specific cell density; and the time it took to attain mid-log phase growth for the specific organisms used in this study.

5.2.2.1 Phosphate Buffer (PBS)

Solution A: 0.1M aqueous solution of potassium phosphate monobasic (KH_2PO_4) was prepared in DI water (10.88g/ 800mL).

Solution B: 0.1 M aqueous solution of potassium phosphate dibasic, anhydrous (K_2HPO_4) was prepared in DI water (13.92g/ 800mL).

A 50mM phosphate buffer saline (PBS) of pH 7 was prepared and used in current assessments by mixing together Solution A (78mL), Solution B (122mL) and DI water (400mL).

5.2.2.2 Antibacterial Assessments

Cell solution preparation: Selected bacteria were inoculated at 37°C in a culture tube containing sterile NB (*ca.* 5mL) for 12 hours. A portion of the resulting culture (*ca.* 1mL) was transferred into a culture flask containing sterile NB (25mL) and incubated at 37°C on a shaker for *ca.* 3.5 hours. A part of the resulting bacterial culture (\approx 2mL) was diluted with sterile NB such that the optical density (OD) of the resulting dilute solution was either 0.400 (for *E. coli*) or 0.800 (for *S. aureus*) when compared to sterile NB as the blank. Once the solution with desired OD was obtained, it was further diluted with four times its volume with sterile NB (e.g. 2mL of bacterial solution of the desired OD + 8mL of sterile NB). The resulting bacterial solution appeared clear to the eye and was immediately stored at 0°C prior to its use. The bacterial cell density in this solution was predetermined to be 4×10^7 cells/mL. This solution was further diluted to 2×10^6 cells in the individual wells during the assessments.

96-well plate preparation: Assessments were made using freshly prepared cell solution as described above. A schematic of the well plate is given in Figure 26. The plate was divided in three distinct categories of columns as shown, with each category containing solutions as indicated below. A combination of Multi-Channel(8) pipettor and sterile troughs was used, when appropriate, to facilitate the plate preparation. One 96-well plate was used to test 5 different agents at one time.

Cell Control: PBS (50 μ L) + Cell solution (50 μ L) + DI water (100 μ L)

Agent Control: PBS (50 μ L) + NB (50 μ L) + Antibacterial agent (100 μ L)[†]

[†] Antibacterial agent was added with varying concentrations along the length of column. For example, across the row 1, the final concentration of the agent in each well, when present, was 256 μ g/mL. Across the (Footnote continued on next page)

Test Well: PBS (50µL) + Cell solution (50µL) + Antibacterial agent (100µL)[‡]

The solutions once introduced into the wells appeared clear and without the presence of any turbidity. The cell count in each of the cell control and test well was calculated to be 2×10^6 cells/well.

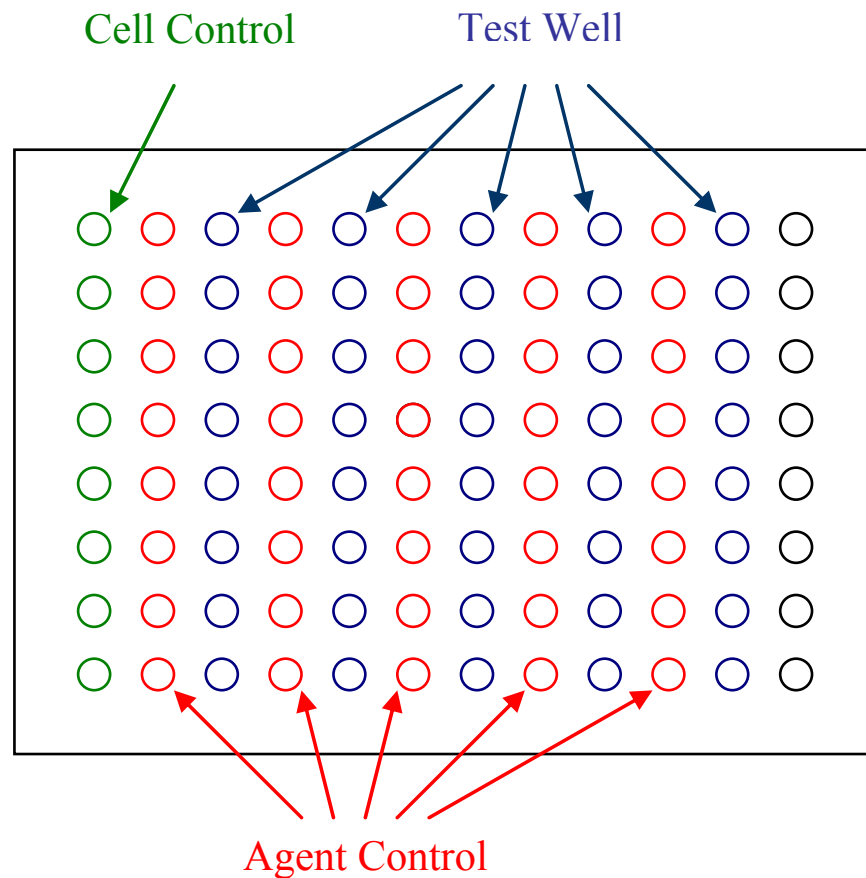


Figure 26: Schematics of a 96-well plate used in antibacterial assessment

Antibacterial assessment: The assessment plate prepared above was incubated at 37°C for 14 hours. After this period, individual results were visually assessed; the wells where bacterial growth occurred turned visibly turbid, indicating no activity against the

row 2, the final concentration was 128µg/mL and so on; until the last row, row 8, which contained a final concentration of the agent at 2µg/mL. Note that cell control did not contain any agent at all.

[‡] see the footnote applicable to section 5.2.2.2.

microorganism growth in such wells. MIC values were assigned as the lowest possible concentration of the agent which inhibited the bacterial growth (identified as the first clear well of lowest agent concentration along the length of each column). Control tests were simultaneously run to ensure; proper bacterial growth within the diluted bacterial culture in the absence of any agent (cell control) and, no growth in solutions of the agent alone in absence of any bacterial culture (agent control).

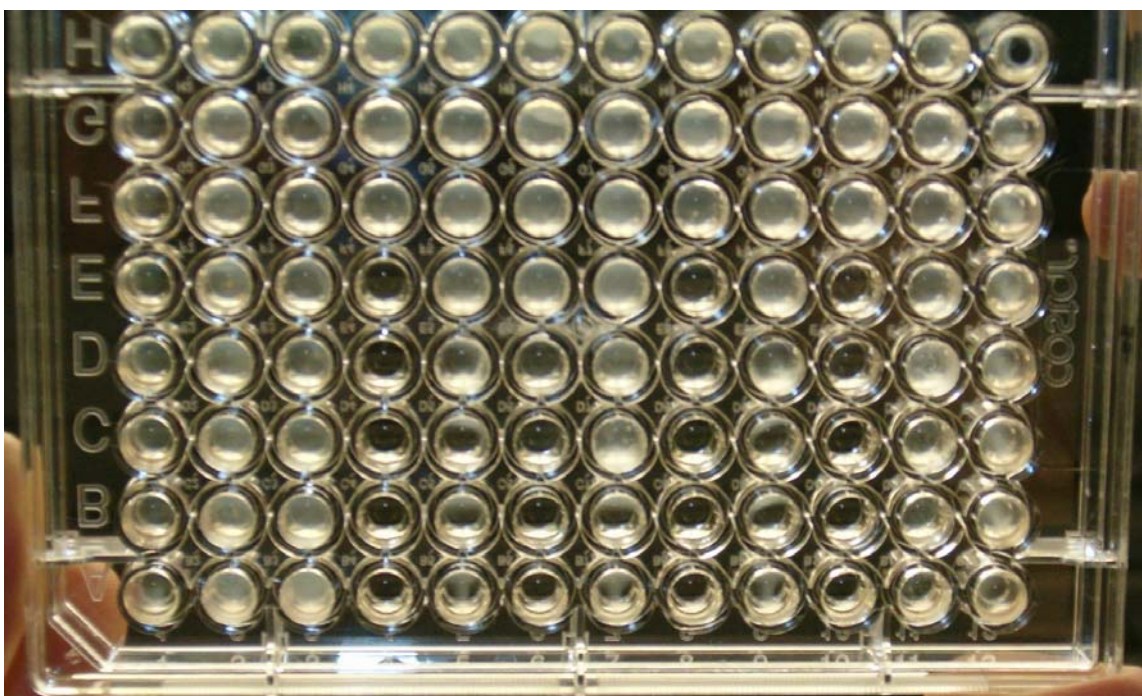


Figure 27: A developed 96-well plate during the antibacterial assessment

Each assessment was performed at least three times to ensure reproducibility of results. The MIC values were accepted when at least two out of the three results were identical. On few occasions, the agent control and test well (Figure 26) were run on two separate plates, but at the same time. A representative plate result is shown in Figure 27. In this particular plate, all the columns contained test wells, except the last one on the right which was cell control. Note the difference in the appearance of the wells that

inhibited bacterial growth (clear, translucent) compared to those which allowed it (marked by the appearance of turbidity).

5.3 Results and Discussion

The MIC values of various chitosan derivatives bearing quaternary ammonium functionality (hence forth referred to as R-Chit-Quat) were determined using *E. coli* (gram-negative) and *S. aureus* (gram-positive) cultures. Higher values of MIC indicate lower antibacterial activity and vice versa. In order to establish the effect of substitution on the activity, the MIC results obtained for R-Chit-Quat were compared with that of quaternized chitosan (referred in here as Chit-Quat). Since the antibacterial assessments were performed using the same method, a qualitative comparison of the effect of various substituents on the activity can be evaluated. Chit-Quat under the assessment protocol we employed, exhibited MIC value of 128 μ g/mL against either bacteria. Most derivatives of chitosan (i.e. R-Chit-Quat) demonstrated an improved antibacterial activity upon introduction of hydrophobic groups compared to this.

5.3.1 Antibacterial Activity Due to Anionic Substituents

Two types of anionic substituents were assessed. The choice of substituent bearing strongly acidic sulfonic acid groups was made in anticipation of its strong polarizability under neutral conditions. It was anticipated that this group would introduce anionic sites with greater overall charge within the polymer backbone. Even with weakly acidic carboxylic acids (i.e. $pK_a \approx 4-5$), at $pH \approx 7$, the degree of dissociation exceeds 98%. The results obtained are given in Table 20. Lower levels of substitution (Table 20, Entries 1, 5 and 9) resulted in activity greater than Chit-Quat (128 μ g/mL). This is attributed to an

increased level of hydrophobic character in these derivatives compared to Chit-Quat.

However, the activity drops with increased amounts of substitution within the same derivative (Table 20, Entries 1-4, 5-8, 9-11).

Table 20: Effect of negatively charged substituents on MIC

Entry	Quat-Derivative *	% substitution	MIC $\mu\text{g/mL}$	
			<i>E. coli</i>	<i>S. aureus</i>
1	2,4-disulf-Bz-Chit-Quat	5.7	32	32
2	2,4-disulf-Bz-Chit-Quat	12.2	>256	>256
3	2,4-disulf-Bz-Chit-Quat	18.0	>256	>256
4	2,4-disulf-Bz-Chit-Quat	24.1	>256	>256
5	2-sulf-Bz-Chit-Quat	8.2	32	32
6	2-sulf-Bz-Chit-Quat	16.9	64	64
7	2-sulf-Bz-Chit-Quat	22.1	256	128
8	2-sulf-Bz-Chit-Quat	30.4	>256	>256
9	4-carbox-Bz-Chit-Quat	8.2	32	32
10	4-carbox-Bz-Chit-Quat	15.6	32	64
11	4-carbox-Bz-Chit-Quat	20.3	>256	>256

* derived from substituted chitosan derivatives shown in Tables 4 and 5.

One of the proposed mechanisms of chitosan-bacteria interaction involves an initial binding thought to occur as a result of electrostatic interactions between the positively charged chitosan macromolecule and negatively charged bacterial cell wall. The adverse effect brought about by chitosan derivatives with higher levels of anionic substitution (Table 20; Entries 2-4, 7-8 and 11) is likely due to the diminished interaction between the macromolecule and the bacterial cell wall. Clearly, the adverse effect on antibacterial activity brought about by the presence of anionic moiety is despite an increase in the hydrophobic character of some derivatives with higher substitutions (Table 20; Entry 11). In case of nonionic derivatives, as will be discussed, introduction of hydrophobic residues

improved the antibacterial activity against both bacteria. Hence, it appears that detrimental effect of anionic substitution on the antibacterial activity is more dominating than the beneficial effect exerted by hydrophobic substitution. In case of *E. coli*, the salts of sulfonic acid substituents seemed to exhibit poor activity compared to those of a weakly acidic carboxylate substituent, at comparable levels of substitution (Table 20; Entries 6 and 10). However, for reasons not clearly understood, this effect was not demonstrated against *S. aureus*. At higher levels of substitution, for derivatives with monosubstituted acidic groups with comparable levels of substitution, *para*-carboxylate moiety exhibited reduced activity than the *ortho*-sulfonate moiety (Table 20; Entries 7 and 11). This reversal in the trend is possibly due to the orientation of these groups on the aromatic ring. A *para* substituent due to its very orientation is farthest away from the substituted nitrogen in chitosan and hence is likely to be more accessible for interaction with bacterial cell components compared to an *ortho* substituent. In general, all anionic substituents past 20% substitution exhibited poor antibacterial activity.

5.3.2 Antibacterial Activity Due to Neutral Hydrophobic Substituents

Chitosan derivatives with neutral hydrophobic substituents were employed in anticipation of facilitated interaction between the antibacterial and the bacterial cell wall due to the presence of hydrophobic groups. The results obtained are given in Table 21. Introducing hydrophobic substituents rendered excellent antibacterial activity to the derivatives (Table 21; Entries 1-12). Chitosan derivatives obtained by γ -lactone addition and anhydride addition (Table 21; Entries 1-6) are particularly interesting, since highly effective derivatives resulted from low levels of substitution. In addition, these

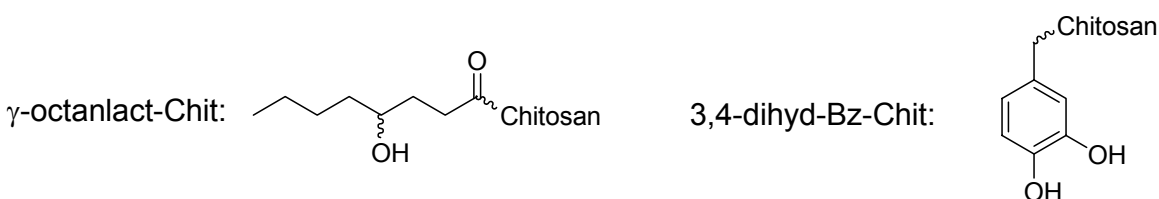
derivatives are of commercial importance since their preparation does not require use of any reducing agent.

Table 21: MIC of chitosan derivatives with neutral substituents

Entry	Quat-Derivative [*]	% substitution	MIC $\mu\text{g/mL}$	
			<i>E. coli</i>	<i>S. aureus</i>
1	γ -octanlact-Chit-Quat	low	32	32
2	γ -octanlact-Chit-Quat	low (but >entry 1)	32	16
3	γ -octanlact-Chit-Quat	low (but >entry 2)	32	32
4	<i>n</i> -heptanoyl-Chit-Quat	1.5	32	32
5	<i>n</i> -heptanoyl-Chit-Quat	1.8	32	32
6	<i>n</i> -heptanoyl-Chit-Quat	2.4	32	32
7	4-acetox-Bz-Chit-Quat	6.3	16	32
8	4-acetox-Bz-Chit-Quat	13.7	32	32
9	4-acetox-Bz-Chit-Quat	19.9	64	32
10	<i>n</i> -octyl-Chit-Quat	4.8	32	32
11	<i>n</i> -octyl-Chit-Quat	5.5	32	32
12	<i>n</i> -octyl-Chit-Quat	11.1	64	128
13	<i>n</i> -octyl-Chit-Quat	35.7	>256	>256
14	Pip-Chit-Quat	12.4	64	128
15	3,4-dihyd-Bz-Chit-Quat	6.4 ^b	32	32
16	3,4-dihyd-Bz-Chit-Quat	25.6 ^b	128	256
17 ^a	3,4-dihyd-Bz-Chit	6.4 ^c	>256	>256

^a chitosan derivative was not quaternized; ^b estimated based on the quat. Derivative;

^c estimated based on entry 15; ^{*} derived from substituted chitosan derivatives shown in Tables 8, 6, 17 and 3.



In the case of γ -octanlact-Chit-Quat (Table 21; Entries 1-3), the levels of substitution were extremely low and as discussed in section 2.4.2, % substitutions in these cases could not be assigned with confidence. One derivative of γ -octanlact-Chit-Quat showed superior activity (Table 21; Entry 2) against *S. aureus* and there could be a favorable effect in place due to the γ -hydroxy group. A favorable effect on antibacterial activity due to a β -hydroxy substituent has been observed in past in the Daly group. Chitosan derivative with *n*-octyl substituent (Table 21; Entries 10-13) exhibited activity that declined with higher levels of substitution. At higher levels of hydrophobic substitution (Table 21; Entry 13), antibacterial activity greatly diminished. A similar effect is observed in the case of 3,4-dihyd-Bz-Chit-Quat (Table 21; Entries 15 and 16). Table 21 clearly indicates that greater hydrophobic character in the quaternized chitosan derivative improved the antibacterial activity. A cross-comparison of the hydrophobic neutral substituents with the anionic substituent indicates the differences in antibacterial activity derived from different substituents. Thus, at comparable levels of substitution, both strongly as well as weakly acidic substituents (Table 20; Entries 7 and 11 respectively) showed much weaker activity compared to the neutral substituent (Table 21; Entry 9). Finally, most of the chitosan derivatives synthesized in the present study were water-insoluble solids at pH 7 prior to their quaternization; except the one with a 3,4-dihydroxy benzyl substituent (Table 21; Entry 17). We were thus able to access the antibacterial activity due to this derivative, containing no quaternary ammonium functionality. The data clearly indicates that presence of quaternary ammonium groups on the hydrophobic chitosan derivatives is essential for the antibacterial activity (Table 21; Entries 15 and 17).

5.3.3 Antibacterial Activity of Chitosan Derived Gels

Different gels prepared in the project were quaternized and their antibacterial activity was assessed. Cross-linkers used in the preparation of these gels have different structural rigidities. Glutaraldehyde derived gels are likely to be more flexible compared to those prepared using terephthalaldehyde.

Table 22: MIC of various chitosan derived gels

Entry	Quaternized Gels *	% CL	% subst.	MIC $\mu\text{g/mL}$	
				<i>E. Coli</i>	<i>S. Aureus</i>
1	CL(tereph)-Chit-Quat	4.8	none	32	16
2	CL(tereph)-Chit-Quat	49.0	none	>256	>256
3	CL(glutar)-Chit-glyox-Quat	(30) ^b	(20) ^b	256	>256
4	CL(tereph)-Chit-glyox-Quat	23.5	(20)	32	64
5	CL(tereph)-Chit-glunh-Quat	8.4	10.6	16	8
6	CL(tereph)-Chit-glunh-Quat	25.4	7.4	32	64
7	CL(tereph)-Chit-glyox-Quat	7.3	(20)	>256	>256
8	CL(tereph)-Chit-glyox-Quat	12.5	(20)	128	128
9 ^a	glunh-Chit-Quat	none	10.1	>256	>256

^a not cross-linked; * derived from gels shown in Tables 18 and 19. ^b estimated values in parenthesis (see section 4.2).

Chitosan gels cross-linked with a hydrophobic cross-linker showed good antibacterial activity at lower degrees of cross-linking (Table 22; Entries 1 and 2). It appears that the rigidity of the resulting macromolecule plays important role in the antibacterial activity, in addition to the hydrophobic character introduced by the cross-linker. Introducing too much rigidity in the macromolecule probably prevents an effective interaction with the bacterial cell over an extended area of its surface. However, cross-linking with a flexible hydrocarbon chain is observed to reduce the antibacterial activity compared to rigid cross-linking (Table 22; Entries 3 and 4). This could be in part due to the differences in

hydrophobicities of an aromatic residue compared to the saturated aliphatic hydrocarbon chain. In addition, hydrophobic cross-linking with a rigid cross-linker greatly enhanced the antibacterial activity of chitosan derivatives with anionic substituents (Table 22; Entries 5 and 9). However, the activity drops with greater cross-linking, despite a decrease in the abundance of carboxylate moieties along the polymer backbone (compare, Table 22; Entries 5 and 6). At a comparable level of cross-linking, substitution with carboxymethyl group resulted in a derivative with greatly reduced activity compared to the derivative with 4-carboxybutyrate substituent (Table 22; Entries 5 and 7). This observation is probably due to the proximity of anionic carboxylic acid moiety to the polymer backbone in former. The further away the anionic group is from the polymer backbone, the lesser its impact on the antibacterial activity. An increase in the level of cross-linking with a rigid hydrophobic cross-linker substantially and progressively countered the reduced antimicrobial activities of derivatives with anionic substituents (Table 22; Entries 7, 8 and 4).

5.4 Summary of Antibacterial Activity Due to Hydrophobic Chitosan Derivatives Bearing Quaternary Ammonium Functionality

Hydrophobic chitosan derivatives bearing quaternary ammonium moieties showed improved antibacterial activity against the representative bacteria *E. coli* and *S. aureus*, compared to the activity of unsubstituted chitosan bearing quaternary ammonium groups (Chit-Quat). It was found that the quaternary groups are essential for the improved activity. Chitosan derivatives with anionic substituents showed reduced activity at higher degrees of substitution. In case of *E. coli*, a strong acid salts (sulfonates) showed lower activity against the bacterium compared to a weak acid salt (carboxylate) at comparable

levels of substitution. Such an effect was not observed against *S. aureus*. Anionic substituents whether weak or strong, generally exhibited very poor activity past 20% substitution; lower than the activity demonstrated by Chit-Quat against both bacteria. In the case of weakly acidic substituents at moderate degrees of substitution, the activity was greatly reinstated upon hydrophobic cross-linking. A rigid hydrophobe at low levels of cross-linking resulted in more effective antibacterial material. Greater levels of cross-linking substantially reduced the antibacterial activity of the derivative. Acyl derivatives resulting from anhydride addition and γ -lactone addition demonstrated excellent antibacterial activity at extremely low levels of substitution. In the case of one such derivative with γ -hydroxy group, the activity was greater against *S. aureus* than *E. coli*. A favorable effect due to the γ -hydroxy group is proposed to explain this difference in the activity, in accordance with the previous observations in the Daly group.

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